Attorney Docket No. 039371-20

IN THE UNITED STATES DESIGNATED/ELECTED OFFICE

In re NEW PATENT Application of)
Kazuo SHINYA et al.)
International Application No. PCT/JP2005/006077)
International Filing Date: 30 March 2005)
For: LABELING SUBSTANCE AND CHIMERA SUBSTANCE, PROCESS FOR PREPARING THESE SUBSTANCES, AND METHOD OF BIOSUBSTANCE TRAPPING, STRUCTURAL ANALYSIS OR/AND IDENTIFICATION WITH USE OF THE LABELING SUBSTANCE)

INFORMATION DISCLOSURE STATEMENT

Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Sir:

In accordance with the duty of disclosure as set forth in 37 C.F.R. §1.56, Applicants hereby submit the following information in conformance with 37 C.F.R. §§ 1.97 and 1.98. Pursuant to 37 C.F.R. § 1.98(a)(2), a copy of the foreign documents cited is enclosed.

A discussion of Japanese Patent Application No. 9-133683 and Japanese Patent Application No. 2002-153272 is given in the specification at pages 3-4.

It is requested that the accompanying information disclosure statement be considered and made of record in the above-captioned application. To assist the Examiner, the documents are listed on the attached form PTO-1449. It is respectfully requested that an Examiner initialed copy of this form be returned to the undersigned.

Respectfully submitted,

Date: September 25, 2006

Thomas Cole

Thomas W. Cole
Registration No. 28,290

Customer No. 25570

Roberts Mlotkowski & Hobbes P.C.
P.O. Box 10064
McLean, VA 22102
Telephone: (703) 677-3001

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U.S. PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Document Number: Number - Kind Code[2] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear
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FOREIGN PATENT DOCUMENTS

Examiner Initials*	Cite No.[1]	Foreign Patent Document: Country Code[3] - Number[4] - Kind Code[5] *(if known)*	Publication Date MM-DD-YYYY	Name of Patentee or Applicant of Cited Document	Pages, Columns, Lines, Where Relevant Passages or Relevant Figures Appear	T[6]
		JP – 9-133683 – A	05-20-1997	Mikio et al.		✓
		JP – 2002-153272 – A	05-28-2002	Hideo et al.		✓
		WO – 95/16209 – A1	06-15-1995	Felder et al.		
		JP – 9-133687 – A	05-20-1997	Toshitada et al.		✓
		EP – 0 488 152 – A2	06-03-1992	Imai et al.		

Examiner Signature		Date Considered	

*EXAMINER: Initial if reference considered, whether or not citation is in conformance with MPEP 609. Draw line through citation if not in conformance and not considered. Include copy of this form with next communication to applicant.

[1] Applicant's unique citation designation number (optional). [2] See Kinds Codes of USPTO Patent Documents at www.uspto.gov or MPEP 901.04. [3] Enter Office that issued the document, by the two-letter code (WIPO Standard ST.3). [4] For Japanese patent documents, the indication of the year of the reign of the Emperor must precede the serial number of the patent document. [5] Kind of document by the appropriate symbols as indicated on the document under WIPO Standard ST.16 if possible. [6] Applicant is to place a check mark here if English language Translation is attached.

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(71)出願人　593184639
中山　幹男
千葉県習志野市鷺沼台3－11－25

(71)出願人　593184628
北野　忠彦
東京都八王子市横川町954－30

(71)出願人　000000527
旭光学工業株式会社
東京都板橋区前野町2丁目36番9号

(72)発明者　中山　幹男
千葉県習志野市鷺沼台3－11－25

(74)代理人　弁理士　三浦　邦夫

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(54)【発明の名称】　抗原又は抗体の検出シート、検出キット及び検出方法

(57)【要約】

【課題】　簡単な操作で高感度で生物学的液体中の抗原又は抗体を検出しうる検出シート、検出キット及び検出方法を提供すること。

【解決手段】　平均粒径が０．０１μｍ～２００μｍでＣａ／Ｐ比が１．０～２．０のリン酸カルシウム系化合物粒子を担持した繊維集合体にアビジン又はストレプトアビジンが固定されていることを特徴とする抗原又は抗体の検出シートである。固定されたアビジン又はストレプトアビジンに、ビオチン化された抗原又は抗体を結合させた後、検出シートを試料溶液と接触させることにより試料中の抗体又は抗原を結合させ、その後、該抗体又は抗原と特異的に結合する標識化合物の溶液と接触させ、標識された抗原－抗体複合体を検出する。

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【特許請求の範囲】

【請求項1】　平均粒径が０．０１μｍ〜２００μｍでＣａ／Ｐ比が１．０〜２．０のリン酸カルシウム系化合物粒子を担持した繊維集合体にアビジン若しくはストレプトアビジン又はこれらの誘導体が固定されていることを特徴とする抗原又は抗体の検出シート。

【請求項2】　固定されたアビジン若しくはストレプトアビジン又はこれらの誘導体に、ビオチン化された抗原又は抗体を結合させた請求項１記載の抗原又は抗体の検出シート。

【請求項3】　繊維集合体の裏面に補強用フィルム又はシートを有する請求項１又は２記載の抗原又は抗体の検出シート。

【請求項4】　請求項２又は３記載の抗原又は抗体の検出シートと標識化合物溶液とからなる抗原又は抗体の検出キット。

【請求項5】　請求項２又は３記載の抗原又は抗体の検出シートを試料溶液と接触させることにより試料中の抗体又は抗原を結合させ、その後、該抗体又は抗原と特異的に結合する標識化合物の溶液と接触させ、標識された抗原−抗体複合体を検出することを特徴とする抗原又は抗体の検出方法。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本発明は、唾液、血液、リンパ液、糞尿などの生物学的液体中の抗原又は抗体を検出する検出シート、検出キット及び検出方法に関する。

【０００２】

【従来の技術】近年、抗原抗体反応を利用した様々な臨床検査が行われているが、特別の施設や臨床検査技師が必要である。そのため、特別の施設がなく、臨床検査技師がいない小病院でも容易に簡単な操作で検査を行うことができ、病気診断の一助にしうる高感度な検査シートが求められている。ハイドロキシアパタイト等のリン酸カルシウム系化合物が蛋白質、核酸などに対して優れた吸着能を有することから、本発明者らは紙や不織布に担持させたリン酸カルシウム系化合物粒子に抗原又は抗体を吸着させ、抗原抗体反応を行わせる検出シートを既に提案している（特願平６−２１４７０６号明細書参照）しかしながら、リン酸カルシウム系化合物は酸性蛋白質に対しては吸着能が不充分であり、また、抗体の吸着に際して吸着部位が必ずしも一定せず、例えば、Ｆabフラグメント部分を吸着することがあるなど、配向性に問題があることが分かった。したがって、リン酸カルシウム系化合物自体では吸着されにくい抗原又は抗体の固定法あるいはリン酸カルシウム系化合物と抗体の有効な結合方法になお改善の余地が残されていた。

【０００３】

【発明が解決しようとする課題】本発明は、上記の従来技術の問題点を解消し、簡単な操作で高感度で生物学的液体中の抗原又は抗体を検出しうる検出シート、検出キット及び検出方法を提供することを目的とする。

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【０００４】

【課題を解決するための手段】本発明は、繊維集合体に担持したリン酸カルシウム系化合物にアビジン又はストレプトアビジンを吸着固定しておくことによって上記目的を達成しうるとの知見に基づいて完成したものである。すなわち、本発明による抗原又は抗体の検出シートは、平均粒径が０．０１μｍ〜２００μｍでＣａ／Ｐ比が１．０〜２．０のリン酸カルシウム系化合物粒子を担持した繊維集合体にアビジン若しくはストレプトアビジン又はこれらの誘導体が固定されていることを特徴とする。

【０００５】本発明の検出シートは、上記のように固定されたアビジン若しくはストレプトアビジン又はこれらの誘導体に、ビオチン化された抗原又は抗体を結合させた状態で提供することもできる。また、本発明による抗原又は抗体の検出方法は、本発明による抗原又は抗体の検出シートを試料溶液と接触させることにより試料中の抗体又は抗原を結合させ、その後、該抗体又は抗原と特異的に結合する標識化合物の溶液と接触させ、標識された抗原−抗体複合体を検出することを特徴とする。

【０００６】

【発明の実施の形態】本発明においては、リン酸カルシウム系化合物にアビジン若しくはストレプトアビジン又はこれらの誘導体を吸着させる。ここで、誘導体としては、アビジンより糖鎖部分を除いたもの、例えばニュートラアビジン（NeutrAvidin ）、ウルトラアビジンなどが挙げられる。

【０００７】本発明においては、アビジン若しくはストレプトアビジン又はこれらの誘導体の吸着固定剤としてリン酸カルシウム系化合物粒子を使用する。ここで、リン酸カルシウム系化合物としては、Ｃａ／Ｐ比が１．０〜２．０であれば各種のリン酸カルシウム系化合物を使用することができ、例えば、$Ca_{10}(PO_4)_6(OH)_2$、$Ca_{10}(PO_4)_6F_2$、$Ca_{10}(PO_4)_6Cl_2$、$Ca_3(PO_4)_2$、$Ca_2P_2O_7$、$Ca_4O(PO_4)_2$ 及び $CaHPO_4$ のうちから選ばれた１種又は２種以上を使用することができる。これらのうちハイドロキシアパタイト及びリン酸三カルシウムが好ましく、特にハイドロキシアパタイトを主成分とするものが最も好ましい。フッ素アパタイトを用いる場合、全リン酸カルシウム系化合物中のフッ素含有率が５重量％以下であるのが好ましい。フッ素含有率が５重量％を超えると、フッ素の溶出が起こり好ましくない。これらのリン酸カルシウム系化合物は、公知の湿式合成法、乾式合成法などによって合成することができる。

【０００８】リン酸カルシウム系化合物の粒子は、例えばリン酸カルシウム系化合物のスラリーを噴霧乾燥することによって造粒し、これを焼成することによって調製

することができるが、この方法に限らず他の造粒法によって調製することも可能である。なお、ふるい分けなどの手段により、粒子の粒度を目的に応じて所定の範囲に選定して用いることがより好ましい。使用するリン酸カルシウム系化合物粒子は、平均粒径が０．０１～２００μｍであるのが好ましい。平均粒径が０．０１μｍ未満であると、粒子が凝集しやすくなり均一に担持されない。また、２００μｍを超えると、不織布に担持しにくくなり、担持率が著しく低下する。

【０００９】さらに、リン酸カルシウム系化合物粒子は、リン酸カルシウム系化合物粒子が比表面積１０m^2／ｇ以上で、細孔径５００～１０００Åの、一次粒子が凝集結合した多孔質粒子であるのが好ましい。比表面積が１０m^2／ｇ未満では充分な吸着能が望めない。また、蛋白質などが吸着されて気孔内へ入り込むためには、細孔径５００～１０００Å程度の気孔を有するのが好ましい。このような多孔質粒子は、任意の公知方法で製造することができる。

【００１０】本発明においては、上記のようなリン酸カルシウム系化合物粒子の担体として繊維集合体を用いる。ここで、使用しうる繊維集合体としては、紙又は不織布が挙げられる。紙にリン酸カルシウム系化合物粒子を担持させる方法としては、リン酸カルシウム系化合物粒子を填料として用い、これを内添方式又は塗工方式により添加して紙を製造する方法がある。内添方式の場合には、リン酸カルシウム系化合物粒子及び他の添加剤を添加し、充分混合した後、通常の抄紙機を用いて製造することができる。また、塗工方式を採用する場合には、結合剤と組み合わせて原紙上に塗布すればよい。結合剤としては、特に制限はなく、例えば、ポリアクリル酸ナトリウム、ポリビニルアルコール、ラテックス、ポリアクリル酸、ポリエチレンオキシド、カルボキシメチルセルロース、ポリエステルなどを使用することができる。

【００１１】不織布にリン酸カルシウム系化合物粒子を担持させる場合にも、紙の場合と同様の方法を採用することができるが、さらに、原料繊維の少なくとも１部分が熱可塑性高分子繊維から成る不織布の少なくとも表面上にリン酸カルシウム系化合物粒子を乾式法又は湿式法で担持させ、次いで、熱処理により不織布中の熱可塑性高分子繊維の少なくとも表面を軟化させて該繊維表面にリン酸カルシウム系化合物粒子を固着させる方法を採用するのが好ましい。

【００１２】繊維集合体へのリン酸カルシウム系化合物の担持量は、通常、１～６５重量%、好ましくは５～５０重量%とするのが好ましい。リン酸カルシウム系化合物の担持量が１重量%未満では、アビジン若しくはストレプトアビジン又はこれらの誘導体を吸着しにくくなり、また、６５重量%を超えるとアビジン若しくはストレプトアビジン又はこれらの誘導体の使用量が高くなるため経済的ではない。

【００１３】こうして製造した繊維集合体の少なくとも表面には、上記のように、リン酸カルシウム系化合物層を有し、リン酸カルシウム系化合物は蛋白質の一種であるアビジン、ストレプトアビジン及びこれらの誘導体に対して高い吸着作用を有し、これらを吸着固定することができる。リン酸カルシウム系化合物を担持した繊維集合体にアビジン若しくはストレプトアビジン又はこれらの誘導体を固定するには、アビジン若しくはストレプトアビジン又はこれらの誘導体の水溶液を用いて浸漬、刷毛塗り、スプレー塗布などの方法で行うことができる。また、アビジン若しくはストレプトアビジン又はこれらの誘導体の固定量は、繊維集合体に担持されているリン酸カルシウム系化合物の種類及び量によって変動し、例えば、ハイドロキシアパタイト３．０ｇ／m^2担持不織布においては、５～２０ｍｇ／m^2が好ましく、１０ｍｇ／m^2前後がより好ましい。５ｍｇ／m^2未満では、ハイドロキシアパタイトの未吸着部位が多く、２０ｍｇ／m^2を超えると、アビジン若しくはストレプトアビジン又はこれらの誘導体量は不経済である。

【００１４】アビジン、ストレプトアビジン及びこれらの誘導体は、ビオチンに対して極めて高い親和性を有し、他方、ビオチンは抗体や多くの酵素に容易に結合させることができるので、上記のように固定されたアビジン若しくはストレプトアビジン又はこれらの誘導体と、ビオチン化した抗原又は抗体とを反応させることにより、繊維集合体上に正しい配向性を以て固定化された抗原又は抗体を有する検査シートが得られ、これを試料溶液と接触させた後、標識化合物溶液と接触させることにより、容易に抗体又は抗原を高感度で検出することができる。

【００１５】本発明においては、必要に応じて、ビオチン化した抗原又は抗体と反応させる前又は反応させた後に、繊維集合体に担持したリン酸カルシウム系化合物粒子の未吸着部位をブロッキング剤でマスキングすることもできるが、この工程は必ずしも必要ではない。また、本発明の検出シートの取扱性の向上のため、検出シートの不織布の裏面に補強用フィルム又はシートを付着させることもできる。補強用フィルム又はシートは、紙又はプラスチックから成るものであってよい。

【００１６】したがって、本発明により、上記のような抗原又は抗体の検出シートと標識化合物溶液とからなる抗原又は抗体の検出キットを提供することができる。標識化合物としては、試料中の抗原又は抗体と特異的に結合する酵素標識抗体又は抗原、同位元素標識抗体又は抗原などを使用することができるが、特別の設備を必要とすることなく、簡単に検出操作を行ないうることから酵素標識が好ましい。酵素標識抗体若しくは抗原を用い、その酵素の可視光又は紫外領域において呈色する基質を用いて目視観察あるいは吸光度測定などの簡単な方法で検出することができる。

【００１７】標識化合物として用いられる酵素と基質としては、例えば、酵素アルカリフォスファターゼに対しては基質ＢＣＩＰ及びＮＢＴ、又はＤＮＰが用いられ、酵素西洋わさびペルオキシダーゼに対しては基質ＯＰＤ、ＤＡＢ又は４ＣＮが用いられ、β－ガラクトシダーゼに対しては基質ｐＮＰＧ、Ｘ－ｇａｌ又はＢｌｕｏ－ｇａｌが用いられる。

【００１８】本発明による検出シート及び検査方法は、主として抗原又は抗体の存否を検出するものであるが、呈色の濃度の比較や吸光度の測定によって抗原又は抗体のある程度の定量も可能である。

【００１９】

【実施例】次に、実施例に基づいて本発明をさらに詳述するが、本発明はこれによって制限されるものではない。

【００２０】実施例１

ポリエチレン５０重量％とポリエチレンテレフタレート５０重量％から成る厚さ０．２ｍｍの不織布にほぼ一様に平均粒径３．５μｍ、Ｃａ／Ｐ比１．６７の多孔質ハイドロキシアパタイト顆粒を加熱処理により２４重量％担持させたアパタイト担持不織布（大きさ５ｍｍ×５ｍｍ）をアビジン５μｇ／ｍｌの水溶液中に浸漬してアビジン１０ｍｇ／ｍ^2 が吸着された不織布を作成し、これにウサギのビオチン化抗ロタ（Rota）ＩｇＧの充分量を結合させ、ロタウイルス検出用検査シートを作成した。得られた検査シートを用いてサンドイッチＥＩＡ法を用いてロタウイルスを検出した。すなわち、ロタウイルス溶液中のロタウイルス抗原を上記検出シートで捕捉する。その後モルモットの抗ロタウイルス・アルカリフォスファターゼ標識抗体を検出シート上のロタウイルス抗原と結合させたのち、ＥＩＡ用基質ＤＮＰの発色により測定した。測定波長は４０５ｎｍであった。ロタウイルス抗原の希釈度を変え、検出シートの感度を検討したところ、０．０６２μｇ／ｍｌのウイルス量まで感知可能であった。上記の測定によるウイルス濃度と吸光度との関係図として図１に示す。

【００２１】比較例１

ポリエチレン５０重量％とポリエチレンテレフタレート５０重量％から成る厚さ０．２ｍｍの不織布にほぼ一様に平均粒径３．５μｍ、Ｃａ／Ｐ比１．６７の多孔質ハイドロキシアパタイト顆粒を加熱処理により２４重量％担持させたアパタイト担持不織布（大きさ５ｍｍ×５ｍｍ）にウサギの抗ロタ（Rota）ＩｇＧ抗体の充分量を吸着させた後、グルタルアルデヒド０．０５％溶液に浸漬処理を行い、上記繊維集合体の抗ロタ抗体未吸着部位をマスキングするためにブロックエース４倍希釈に浸漬して、ロタウイルス検出シートを作成した。得られたロタウイルス検出シートを用いて、実施例１と同様の検出条件で、ロタウイルス０．２５μｇ／ｍｌの検出を試みたが、吸光度は０．０１であり、検出できなかった。一方、実施例１によって作成された検出シートを用いてロタウイルス０．２５μｇ／ｍｌの検出を行なったところ、吸光度は０．９５であり、充分に検出を行なうことができた。

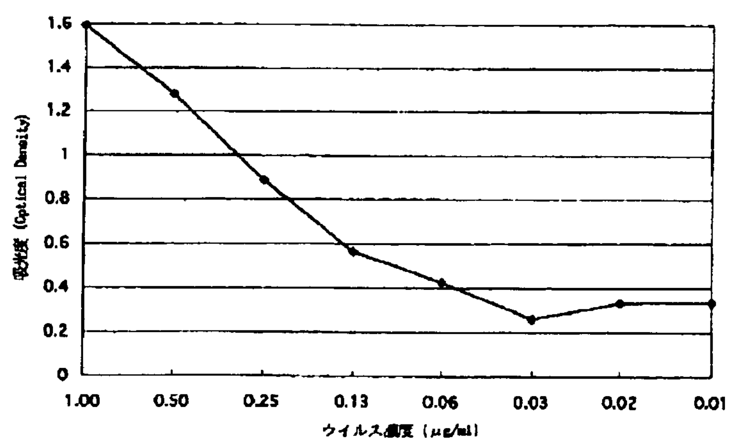
【０００２２】

【発明の効果】本発明の検出シートを用いれば、固定化すべき抗原や抗体の種類に制限がなく、また、配向性の問題もなく、簡単な操作で、迅速に高感度で生物学的液体中の抗原又は抗体を検出することができ、検査技師などの熟練者のいない小規模病院でも抗原又は抗体の検出を容易に行ないうる安価で高感度な検出キットを提供することができる。

【図面の簡単な説明】

【図１】実施例１におけるロタウイルスの検出結果を示すグラフ図である。

【図１】



フロントページの続き

(72)発明者　北野　忠彦
東京都八王子市横川町954－30
(72)発明者　見藤　歩
東京都板橋区前野町2丁目36番9号　旭光学工業株式会社内
(72)発明者　小川　哲朗
東京都板橋区前野町2丁目36番9号　旭光学工業株式会社内
(72)発明者　平出　恒男
東京都板橋区前野町2丁目36番9号　旭光学工業株式会社内

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(71)Applicant : NAKAYAMA MIKIO
KITANO TADAHIKO
ASAHI OPTICAL CO LTD

(72)Inventor : NAKAYAMA MIKIO
KITANO TADAHIKO
KENDO AYUMI
OGAWA TETSURO
HIRAIDE TSUNEO

(54) SHEET, KIT, AND METHOD FOR DETECTING ANTIGEN OR ANTIBODY

(57)Abstract:
PROBLEM TO BE SOLVED: To provide a sheet, kit, and method for detecting antigen or antibody by which an antigen or antibody contained in a biological solution can be detected with high sensitivity through simple operation.
SOLUTION: A sheet for detecting antigen or antibody is constituted by immobilizing avidin or streptavidin to a fiber aggregate carrying particles of a calcium phosphate compound having an average particle diameter of 0.01-200μm and a Ca/P ratio of 1.0-2.0. By bonding an antibody or antigen contained in a sample solution to the immobilized avidin or streptoavidin by bringing the sheet into contact with the sample solution after bonding a biotinized antigen or antibody to the avidin or streptoavidin and bringing the sheet into contact with the solution of a labeled compound which is specifically bonded to the antibody or antigen in the sample solution, a labeled antigen- antibody complex is detected.

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* NOTICES *

CLAIMS

[Claim(s)]
[Claim 1] The detection sheet of the antigen or antibody characterized by fixing avidin, streptoavidin, or these derivatives to the fiber aggregate with which the calcium/P ratio supported [mean particle diameter] the calcium phosphate system compound particle of 1.0-2.0 with 0.01 micrometers - 200 micrometers.
[Claim 2] The antigen according to claim 1 which combined the antigen or antibody biotin-ized with the fixed avidin, streptoavidin, or these derivatives, or the detection sheet of an antibody.
[Claim 3] The antigen according to claim 1 or 2 which has a film for reinforcement, or a sheet at the rear face of the fiber aggregate, or the detection sheet of an antibody.
[Claim 4] The detection kit of the antigen or antibody which consists of an antigen according to claim 2 or 3, or the detection sheet and labeled compound solution of an antibody.
[Claim 5] The detection approach of of the antigen or antibody characterized by detecting the antigen-antibody complex by which was made to combine the antibody or antigen in a sample, and was contacted in the solution of the labeled compound specifically combined with this antibody or an antigen after that, and the indicator was carried out by contacting an antigen according to claim 2 or 3 or the detection sheet of an antibody to the sample solution.

[Translation done.]

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DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] This invention relates to the detection sheet, detection kit, and the detection approach of detecting the antigen or antibody in biological liquids, such as saliva, blood, lymph, and feces and urine.
[0002]
[Description of the Prior Art] Although various clinical laboratory tests using an antigen-antibody reaction are conducted in recent years, a special facility and a special clinical laboratory technologist are required. Therefore, there is no special facility, it can inspect by easy actuation easily also in the small hospital in which a clinical laboratory technologist is not present, and the high sensitivity inspection sheet which can be made into an aid of a sick diagnosis is called for. Since it has the adsorption capacity in which calcium phosphate system compounds, such as hydroxyapatite, were excellent to protein, a nucleic acid, etc., this invention persons made the antigen or the antibody stick to the calcium phosphate system compound particle which paper and a nonwoven fabric were made to support, and the detection sheet on which an antigen-antibody reaction is made to perform has already been proposed (refer to Japanese-Patent-Application-No. No. 214706 [six to] specification).
However, it turned out that a problem is in that a calcium phosphate system compound has inadequate adsorption capacity, its adsorption site may not necessarily be fixed on the occasion of adsorption of an antibody to acidic protein, for example, a Fab fragment part may be adsorbed, and a stacking tendency. Therefore, in addition, the room of an improvement was left behind to the fixing method of the antigen which is hard to adsorb, or an antibody, or the effective joint approach of a calcium phosphate system compound and an antibody with the calcium phosphate system compound itself.
[0003]
[Problem(s) to be Solved by the Invention] This invention cancels the trouble of the above-mentioned conventional technique, and aims at offering the detection sheet, detection kit, and the detection approach high sensitivity can detect the antigen or antibody in a biological liquid by easy actuation.
[0004]
[Means for Solving the Problem] This invention is completed based on knowledge that the above-mentioned purpose can be attained by carrying out adsorption immobilization of avidin or the streptoavidin to the calcium phosphate system compound supported to the fiber aggregate. That is, the antigen by this invention or the detection sheet of an antibody is characterized by fixing avidin, streptoavidin, or these derivatives to the fiber aggregate with which the calcium/P ratio supported [mean particle diameter] the calcium phosphate system compound particle of 1.0-2.0 with 0.01 micrometers - 200 micrometers.
[0005] The detection sheet of this invention can also be offered where the antigen or antibody biotin-ized is combined with the avidin fixed as mentioned above, streptoavidin, or these derivatives. Moreover, by contacting the antigen by this invention, or the detection sheet of an antibody to the sample solution, the detection approach of the antigen by this invention or an

antibody combines the antibody or antigen in a sample, is contacted in the solution of the labeled compound specifically combined with this antibody or an antigen after that, and is characterized by detecting the antigen-antibody complex by which the indicator was carried out.
[0006]
[Embodiment of the Invention] Avidin, streptoavidin, or these derivatives are made to stick to a calcium phosphate system compound in this invention. Here, as a derivative, the thing (NeutrAvidin), for example, new trad avidin, excluding the sugar chain part from avidin, ultra avidin, etc. are mentioned.
[0007] In this invention, a calcium phosphate system compound particle is used as avidin, streptoavidin, or an adsorption fixative of these derivatives. Here as a calcium phosphate system compound If calcium/P ratios are 1.0-2.0, various kinds of calcium phosphate system compounds can be used. For example, calcium10(PO4) 6(OH)2 and calcium10(PO4) 6 F2, calcium10(PO4) 6 Cl2, calcium3(PO4) 2, calcium two P2O7, and calcium 4O(PO4) 2 And CaHPO4 One sort chosen from inside or two sorts or more can be used. Hydroxyapatite and tricalcium phosphate are [among these] desirable, and what uses especially hydroxyapatite as a principal component is the most desirable. When using a fluorine apatite, it is desirable that the fluorine content in a total phosphorus acid calcium system compound is 5 or less % of the weight. The elution of a fluorine happens and is not desirable if fluorine content exceeds 5 % of the weight. These calcium phosphate system compounds are compoundable with a well-known wet synthesis method, a dry type synthesis method, etc.
[0008] Although it can prepare by corning the particle of a calcium phosphate system compound by carrying out spray drying of the slurry of for example, a calcium phosphate system compound, and calcinating this, not only this approach but the thing to prepare by other corning methods is possible. In addition, it is more desirable to select and use for the predetermined range [for the purpose of the grain size of a particle] with means, such as sieving. As for the calcium phosphate system compound particle to be used, it is desirable that mean particle diameter is 0.01-200 micrometers. It becomes it easy to condense a particle that mean particle diameter is less than 0.01 micrometers, and is not supported by homogeneity. Moreover, if it exceeds 200 micrometers, it will be hard coming to support to a nonwoven fabric, and the rate of support will fall remarkably.
[0009] Furthermore, calcium phosphate system compound particles are more than specific surface area of 10m 2 / g, and, as for a calcium phosphate system compound particle, it is desirable that it is the porosity particle in which the primary particle with a pole diameter of 500-1000A carried out condensation association. Adsorption capacity with a specific surface area sufficient by under 10m2 / g cannot be desired. Moreover, in order to adsorb and for protein etc. to enter into pore, it is desirable to have pore with a pole diameter of about 500-1000A. Such a porosity particle can be manufactured by the well-known approach of arbitration.
[0010] In this invention, the fiber aggregate is used as support of the above calcium phosphate system compound particles. Here, paper or a nonwoven fabric is mentioned as the fiber aggregate which can be used. There is a method of adding this with an inner attachment method or a coating method as an approach of making paper supporting a calcium phosphate system compound particle, using a calcium phosphate system compound particle as a loading material, and manufacturing paper. In the case of an inner attachment method, after adding a calcium phosphate system compound particle and other additives and mixing enough, it can manufacture using the usual paper machine. Moreover, what is necessary is just to apply in the Hara paper combining a binder, in adopting a coating method. As a binder, there is especially no limit, for example, it can use sodium polyacrylate, polyvinyl alcohol, a latex, polyacrylic acid, polyethylene oxide, a carboxymethyl cellulose, polyester, etc.
[0011] Although the same approach as the case of paper can be adopted also when making a nonwoven fabric support a calcium phosphate system compound particle Even if there are few nonwoven fabrics with which at least 1 part of raw material fiber consists of thermoplastic macromolecule fiber, a calcium phosphate system compound particle is made to support with dry process or a wet method on a front face. Furthermore, subsequently It is desirable to adopt the approach of the thermoplastic macromolecule fiber in a nonwoven fabric of softening a front face

at least and making this fiber front face fixing a calcium phosphate system compound particle, by heat treatment.
[0012] As for the amount of support of the calcium phosphate system compound to the fiber aggregate, it is usually preferably desirable to consider as 5 - 50 % of the weight one to 65% of the weight. At less than 1 % of the weight, since the amount of avidin, streptoavidin, or these derivatives used will become high if it is hard coming to adsorb avidin, streptoavidin, or these derivatives and exceeds 65 % of the weight, the amount of support of a calcium phosphate system compound is not economical.
[0013] In this way, it has a high absorption to the avidin of the manufactured fiber aggregate whose calcium phosphate system compound it has a calcium phosphate system compound layer as mentioned above in a front face, and is a proteinic kind at least, streptoavidin, and these derivatives, and adsorption immobilization of these can be carried out. In order to fix avidin, streptoavidin, or these derivatives to the fiber aggregate which supported the calcium phosphate system compound, it can carry out by the approach of immersion, brush coating, a spray coating cloth, etc. using the water solution of avidin, streptoavidin, or these derivatives. Moreover, avidin, streptoavidin, or the amount of immobilization of these derivatives is changed with the class and amount of a calcium phosphate system compound which are supported by the fiber aggregate, for example, is hydroxyapatite 3.0 g/m2. It sets to a support nonwoven fabric and is 5 - 20 mg/m2. It is desirable and is 10 mg/m2. Order is more desirable. 5 mg/m2 There are many non-adsorption sites of hydroxyapatite at the following, and it is 20 mg/m2. If it exceeds, avidin, streptoavidin, or these amounts of derivatives are uneconomical.
[0014] Avidin, streptoavidin, and these derivatives Since it has very high compatibility to a biotin and another side and a biotin can be easily combined with the enzyme of an antibody or many By making the avidin fixed as mentioned above, streptoavidin or these derivatives, and the antigen or antibody biotin-ized react a fiber aggregate top -- a right stacking tendency -- with, after obtaining the inspection sheet which has the fixed antigen or antibody and contacting this to the sample solution, an antibody or an antigen is easily detectable by high sensitivity by making a labeled compound solution contact.
[0015] In this invention, although the antigen or antibody biotin-ized if needed, and the front stirrup made to react can also mask the non-adsorption site of the calcium phosphate system compound particle supported to the fiber aggregate by the blocking agent after making it react, this process is not necessarily required. Moreover, the film for reinforcement or a sheet can also be made to adhere to the rear face of the nonwoven fabric of a detection sheet for improvement in the handling nature of the detection sheet of this invention. The film for reinforcement or a sheet may consist of paper or plastics.
[0016] Therefore, the detection kit of the antigen or antibody which consists of the above antigens, or the detection sheet and labeled compound solution of an antibody can be offered by this invention. Although the enzyme labelled antibody specifically combined with the antigen in a sample or an antibody as a labeled compound or an antigen, an isotope labelled antibody, or an antigen can be used, since detection actuation can be performed easily, enzyme labeling is desirable, without needing a special facility. It is detectable by easy approaches, such as visual observation or spectrometry, using the substrate which carries out coloration in the light or the ultraviolet region of the enzyme using an enzyme labelled antibody or an antigen.
[0017] As the enzyme used as a labeled compound, and a substrate, Substrates BCIP, NBT, or DNP are used to enzyme alkaline phosphatase, Substrates OPD and DAB or 4CN(s) are used to enzyme horseradish peroxidase, and Substrate pNPG, X-gal, or Bluo-gal is used to the beta-galactosidase, for example.
[0018] Although the detection sheet and the inspection approach by this invention mainly detect the existence or nonexistence of an antigen or an antibody, a certain amount of quantum of an antigen or an antibody is also possible for them by the comparison of the concentration of coloration, or measurement of an absorbance.
[0019]
[Example] Next, this invention is not restricted by this although this invention is further explained in full detail based on an example.

[0020] At about 1 appearance to a nonwoven fabric with a thickness of 0.2mm which consists of 50 % of the weight of example 1 polyethylene, and 50 % of the weight of polyethylene terephthalate The mean particle diameter of 3.5 micrometers, The apatite support nonwoven fabric (magnitude 5mmx5mm) which made the porosity hydroxyapatite granulation of the calcium/P ratio 1.67 support 24% of the weight by heat-treatment is immersed into an avidin 5microg/ml water solution, and it is avidin 10 mg/m2. The adsorbed nonwoven fabric is created. this -- biotin-ized anti-Rota (Rota) IgG of a rabbit -- the amount was combined enough and the inspection sheet for rotavirus detection was created. the obtained inspection sheet -- using -- Sandwiches EIA -- the rotavirus was detected using law. That is, the rotavirus antigen in a rotavirus solution is caught with the above-mentioned detection sheet. After combining the anti-rotavirus alkaline phosphatase labelled antibody of a guinea pig with the rotavirus antigen on a detection sheet after that, it measured by coloring of the substrate DNP for EIA. Measurement wavelength was 405nm. When the dilution of a rotavirus antigen was changed and the sensibility of a detection sheet was examined, it has sensed to the amount of 0.062microg [/ml] viruses. It is shown in drawing 1 as a related Fig. of the virus concentration and the absorbance by the above-mentioned measurement.

[0021] At about 1 appearance to a nonwoven fabric with a thickness of 0.2mm which consists of 50 % of the weight of example of comparison 1 polyethylene, and 50 % of the weight of polyethylene terephthalate The mean particle diameter of 3.5 micrometers, The porosity hydroxyapatite granulation of the calcium/P ratio 1.67 to the apatite support nonwoven fabric (magnitude 5mmx5mm) made to support 24% of the weight by heat-treatment After [the anti-Rota (Rota) IgG antibody of a rabbit] making an amount adsorb enough, Immersion processing was performed in the glutaraldehyde 0.05% solution, in order to mask the anti-Rota antibody non-adsorption site of the above-mentioned fiber aggregate, it was immersed in 4 times many block ace [as this] dilution, and the rotavirus detection sheet was created. Although detection of rotavirus 0.25microg/ml was tried on the same detection conditions as an example 1 using the obtained rotavirus detection sheet, an absorbance is 0.01 and was not able to be detected. On the other hand, when detection of rotavirus 0.25microg/ml was performed using the detection sheet created by the example 1, an absorbance is 0.95 and was fully detectable.

[0022]

[Effect of the Invention] If the detection sheet of this invention is used, there is no limit in the class of the antigen which should be fixed, or antibody, and there is also no problem of a stacking tendency, by easy actuation, the antigen or antibody in a biological liquid can be quickly detected by high sensitivity, and the detection kit [that it is cheap and high sensitivity] which can perform detection of an antigen or an antibody easily also in the small-scale hospital in which experts, such as a laboratory technician, are not present can be offered.

[Translation done.]

TECHNICAL FIELD

[Field of the Invention] This invention relates to the detection sheet, detection kit, and the detection approach of detecting the antigen or antibody in biological liquids, such as saliva, blood, lymph, and feces and urine.

[Translation done.]

* NOTICES *

PRIOR ART

[Description of the Prior Art] Although various clinical laboratory tests using an antigen-antibody reaction are conducted in recent years, a special facility and a special clinical laboratory technologist are required. Therefore, there is no special facility, it can inspect by easy actuation easily also in the small hospital in which a clinical laboratory technologist is not present, and the high sensitivity inspection sheet which can be made into an aid of a sick diagnosis is called for. Since it has the adsorption capacity in which calcium phosphate system compounds, such as hydroxyapatite, were excellent to protein, a nucleic acid, etc., this invention persons made the antigen or the antibody stick to the calcium phosphate system compound particle which paper and a nonwoven fabric were made to support, and the detection sheet on which an antigen-antibody reaction is made to perform has already been proposed (refer to Japanese-Patent-Application-No. No. 214706 [six to] specification).
However, it turned out that a problem is in that a calcium phosphate system compound has inadequate adsorption capacity, its adsorption site may not necessarily be fixed on the occasion of adsorption of an antibody to acidic protein, for example, a Fab fragment part may be adsorbed, and a stacking tendency. Therefore, in addition, the room of an improvement was left behind to the fixing method of the antigen which is hard to adsorb, or an antibody, or the effective joint approach of a calcium phosphate system compound and an antibody with the calcium phosphate system compound itself.

[Translation done.]

EFFECT OF THE INVENTION

[Effect of the Invention] If the detection sheet of this invention is used, there is no limit in the class of the antigen which should be fixed, or antibody, and there is also no problem of a stacking tendency, by easy actuation, the antigen or antibody in a biological liquid can be quickly detected by high sensitivity, and the detection kit [that it is cheap and high sensitivity] which can perform detection of an antigen or an antibody easily also in the small-scale hospital in which experts, such as a laboratory technician, are not present can be offered.

[Translation done.]

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] This invention cancels the trouble of the above-mentioned conventional technique, and aims at offering the detection sheet, detection kit, and the detection approach high sensitivity can detect the antigen or antibody in a biological liquid by easy actuation.

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MEANS

[Means for Solving the Problem] This invention is completed based on knowledge that the above-mentioned purpose can be attained by carrying out adsorption immobilization of avidin or the streptoavidin to the calcium phosphate system compound supported to the fiber aggregate. That is, the antigen by this invention or the detection sheet of an antibody is characterized by fixing avidin, streptoavidin, or these derivatives to the fiber aggregate with which the calcium/P ratio supported [mean particle diameter] the calcium phosphate system compound particle of 1.0-2.0 with 0.01 micrometers - 200 micrometers.
[0005] The detection sheet of this invention can also be offered where the antigen or antibody biotin-ized is combined with the avidin fixed as mentioned above, streptoavidin, or these derivatives. Moreover, by contacting the antigen by this invention, or the detection sheet of an antibody to the sample solution, the detection approach of the antigen by this invention or an antibody combines the antibody or antigen in a sample, is contacted in the solution of the labeled compound specifically combined with this antibody or an antigen after that, and is characterized by detecting the antigen-antibody complex by which the indicator was carried out.
[0006]
[Embodiment of the Invention] Avidin, streptoavidin, or these derivatives are made to stick to a calcium phosphate system compound in this invention. Here, as a derivative, the thing (NeutrAvidin), for example, new trad avidin, excluding the sugar chain part from avidin, ultra avidin, etc. are mentioned.
[0007] In this invention, a calcium phosphate system compound particle is used as avidin, streptoavidin, or an adsorption fixative of these derivatives. Here as a calcium phosphate system compound If calcium/P ratios are 1.0-2.0, various kinds of calcium phosphate system compounds can be used. For example, calcium10(PO4) 6(OH)2 and calcium10(PO4) 6 F2, calcium10(PO4) 6 Cl2, calcium3(PO4) 2, calcium two P2O7, and calcium 4O(PO4) 2 And CaHPO4 One sort chosen from inside or two sorts or more can be used. Hydroxyapatite and tricalcium phosphate are [among these] desirable, and what uses especially hydroxyapatite as a principal component is the most desirable. When using a fluorine apatite, it is desirable that the fluorine content in a total phosphorus acid calcium system compound is 5 or less % of the weight. The elution of a fluorine happens and is not desirable if fluorine content exceeds 5 % of the weight. These calcium phosphate system compounds are compoundable with a well-known wet synthesis method, a dry type synthesis method, etc.
[0008] Although it can prepare by corning the particle of a calcium phosphate system compound by carrying out spray drying of the slurry of for example, a calcium phosphate system compound, and calcinating this, not only this approach but the thing to prepare by other corning methods is possible. In addition, it is more desirable to select and use for the predetermined range [for the purpose of the grain size of a particle] with means, such as sieving. As for the calcium phosphate system compound particle to be used, it is desirable that mean particle diameter is 0.01-200 micrometers. It becomes it easy to condense a particle that mean particle diameter is less than 0.01 micrometers, and is not supported by homogeneity. Moreover, if it exceeds 200 micrometers, it will be hard coming to support to a nonwoven fabric, and the rate of support will fall remarkably.

[0009] Furthermore, calcium phosphate system compound particles are more than specific surface area of 10m 2 / g, and, as for a calcium phosphate system compound particle, it is desirable that it is the porosity particle in which the primary particle with a pole diameter of 500-1000A carried out condensation association. Adsorption capacity with a specific surface area sufficient by under 10m2 / g cannot be desired. Moreover, in order to adsorb and for protein etc. to enter into pore, it is desirable to have pore with a pole diameter of about 500-1000A. Such a porosity particle can be manufactured by the well-known approach of arbitration.
[0010] In this invention, the fiber aggregate is used as support of the above calcium phosphate system compound particles. Here, paper or a nonwoven fabric is mentioned as the fiber aggregate which can be used. There is a method of adding this with an inner attachment method or a coating method as an approach of making paper supporting a calcium phosphate system compound particle, using a calcium phosphate system compound particle as a loading material, and manufacturing paper. In the case of an inner attachment method, after adding a calcium phosphate system compound particle and other additives and mixing enough, it can manufacture using the usual paper machine. Moreover, what is necessary is just to apply in the Hara paper combining a binder, in adopting a coating method. As a binder, there is especially no limit, for example, it can use sodium polyacrylate, polyvinyl alcohol, a latex, polyacrylic acid, polyethylene oxide, a carboxymethyl cellulose, polyester, etc.
[0011] Although the same approach as the case of paper can be adopted also when making a nonwoven fabric support a calcium phosphate system compound particle Even if there are few nonwoven fabrics with which at least 1 part of raw material fiber consists of thermoplastic macromolecule fiber, a calcium phosphate system compound particle is made to support with dry process or a wet method on a front face. Furthermore, subsequently It is desirable to adopt the approach of the thermoplastic macromolecule fiber in a nonwoven fabric of softening a front face at least and making this fiber front face fixing a calcium phosphate system compound particle, by heat treatment.
[0012] As for the amount of support of the calcium phosphate system compound to the fiber aggregate, it is usually preferably desirable to consider as 5 - 50 % of the weight one to 65% of the weight. At less than 1 % of the weight, since the amount of avidin, streptoavidin, or these derivatives used will become high if it is hard coming to adsorb avidin, streptoavidin, or these derivatives and exceeds 65 % of the weight, the amount of support of a calcium phosphate system compound is not economical.
[0013] In this way, it has a high absorption to the avidin of the manufactured fiber aggregate whose calcium phosphate system compound it has a calcium phosphate system compound layer as mentioned above in a front face, and is a proteinic kind at least, streptoavidin, and these derivatives, and adsorption immobilization of these can be carried out. In order to fix avidin, streptoavidin, or these derivatives to the fiber aggregate which supported the calcium phosphate system compound, it can carry out by the approach of immersion, brush coating, a spray coating cloth, etc. using the water solution of avidin, streptoavidin, or these derivatives. Moreover, avidin, streptoavidin, or the amount of immobilization of these derivatives is changed with the class and amount of a calcium phosphate system compound which are supported by the fiber aggregate, for example, is hydroxyapatite 3.0 g/m2. It sets to a support nonwoven fabric and is 5 - 20 mg/m2. It is desirable and is 10 mg/m2. Order is more desirable. 5 mg/m2 There are many non-adsorption sites of hydroxyapatite at the following, and it is 20 mg/m2. If it exceeds, avidin, streptoavidin, or these amounts of derivatives are uneconomical.
[0014] Avidin, streptoavidin, and these derivatives Since it has very high compatibility to a biotin and another side and a biotin can be easily combined with the enzyme of an antibody or many By making the avidin fixed as mentioned above, streptoavidin or these derivatives, and the antigen or antibody biotin-ized react a fiber aggregate top -- a right stacking tendency -- with, after obtaining the inspection sheet which has the fixed antigen or antibody and contacting this to the sample solution, an antibody or an antigen is easily detectable by high sensitivity by making a labeled compound solution contact.
[0015] In this invention, although the antigen or antibody biotin-ized if needed, and the front stirrup made to react can also mask the non-adsorption site of the calcium phosphate system

compound particle supported to the fiber aggregate by the blocking agent after making it react, this process is not necessarily required. Moreover, the film for reinforcement or a sheet can also be made to adhere to the rear face of the nonwoven fabric of a detection sheet for improvement in the handling nature of the detection sheet of this invention. The film for reinforcement or a sheet may consist of paper or plastics.

[0016] Therefore, the detection kit of the antigen or antibody which consists of the above antigens, or the detection sheet and labeled compound solution of an antibody can be offered by this invention. Although the enzyme labelled antibody specifically combined with the antigen in a sample or an antibody as a labeled compound or an antigen, an isotope labelled antibody, or an antigen can be used, since detection actuation can be performed easily, enzyme labeling is desirable, without needing a special facility. It is detectable by easy approaches, such as visual observation or spectrometry, using the substrate which carries out coloration in the light or the ultraviolet region of the enzyme using an enzyme labelled antibody or an antigen.

[0017] As the enzyme used as a labeled compound, and a substrate, Substrates BCIP, NBT, or DNP are used to enzyme alkaline phosphatase, Substrates OPD and DAB or 4CN(s) are used to enzyme horseradish peroxidase, and Substrate pNPG, X-gal, or Bluo-gal is used to the beta-galactosidase, for example.

[0018] Although the detection sheet and the inspection approach by this invention mainly detect the existence or nonexistence of an antigen or an antibody, a certain amount of quantum of an antigen or an antibody is also possible for them by the comparison of the concentration of coloration, or measurement of an absorbance.

[Translation done.]

EXAMPLE

[Example] Next, this invention is not restricted by this although this invention is further explained in full detail based on an example.
[0020] At about 1 appearance to a nonwoven fabric with a thickness of 0.2mm which consists of 50 % of the weight of example 1 polyethylene, and 50 % of the weight of polyethylene terephthalate The mean particle diameter of 3.5 micrometers, The apatite support nonwoven fabric (magnitude 5mmx5mm) which made the porosity hydroxyapatite granulation of the calcium/P ratio 1.67 support 24% of the weight by heat-treatment is immersed into an avidin 5microg/ml water solution, and it is avidin 10 mg/m2. The adsorbed nonwoven fabric is created. this -- biotin-ized anti-Rota (Rota) IgG of a rabbit -- the amount was combined enough and the inspection sheet for rotavirus detection was created. the obtained inspection sheet -- using -- Sandwiches EIA -- the rotavirus was detected using law. That is, the rotavirus antigen in a rotavirus solution is caught with the above-mentioned detection sheet. After combining the anti-rotavirus alkaline phosphatase labelled antibody of a guinea pig with the rotavirus antigen on a detection sheet after that, it measured by coloring of the substrate DNP for EIA. Measurement wavelength was 405nm. When the dilution of a rotavirus antigen was changed and the sensibility of a detection sheet was examined, it has sensed to the amount of 0.062microg [/ml] viruses. It is shown in drawing 1 as a related Fig. of the virus concentration and the absorbance by the above-mentioned measurement.
[0021] At about 1 appearance to a nonwoven fabric with a thickness of 0.2mm which consists of 50 % of the weight of example of comparison 1 polyethylene, and 50 % of the weight of polyethylene terephthalate The mean particle diameter of 3.5 micrometers, The porosity hydroxyapatite granulation of the calcium/P ratio 1.67 to the apatite support nonwoven fabric (magnitude 5mmx5mm) made to support 24% of the weight by heat-treatment After [the anti-Rota (Rota) IgG antibody of a rabbit] making an amount adsorb enough, Immersion processing was performed in the glutaraldehyde 0.05% solution, in order to mask the anti-Rota antibody non-adsorption site of the above-mentioned fiber aggregate, it was immersed in 4 times many block ace [as this] dilution, and the rotavirus detection sheet was created. Although detection of rotavirus 0.25microg/ml was tried on the same detection conditions as an example 1 using the obtained rotavirus detection sheet, an absorbance is 0.01 and was not able to be detected. On the other hand, when detection of rotavirus 0.25microg/ml was performed using the detection sheet created by the example 1, an absorbance is 0.95 and was fully detectable.

[Translation done.]

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is the graphical representation showing the detection result of the rotavirus in an example 1.

[Translation done.]

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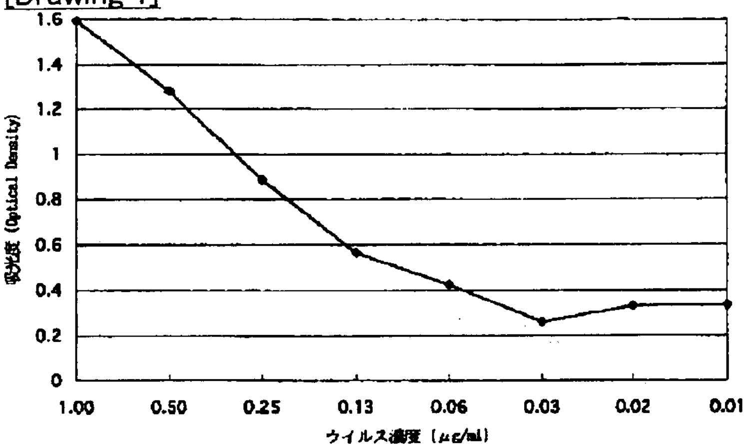
DRAWINGS

[Drawing 1]



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(71)出願人　000006792
理化学研究所
埼玉県和光市広沢２番１号

(71)出願人　390001421
学校法人早稲田大学
東京都新宿区戸塚町１丁目104番地

(72)発明者　田代　英夫
埼玉県和光市広沢２番１号　理化学研究所内

(74)代理人　100085660
弁理士　鈴木　均

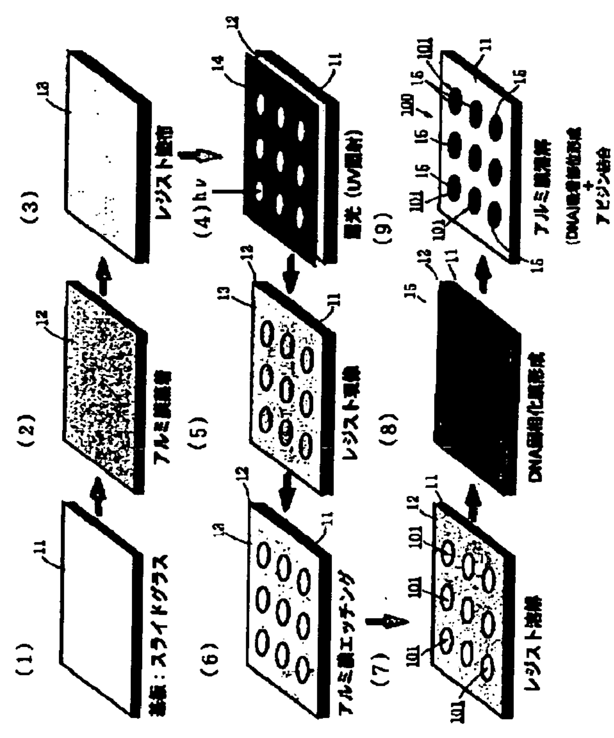
最終頁に続く

(54)【発明の名称】　生体分子マイクロアレイ

(57)【要約】

【課題】　定量的な解析に使用できる、Ｓ／Ｎ比の高い生体分子マイクロアレイを提供する。

【解決手段】　フォトリソグラフィ技術およびエッチング技術を用いて、スライドグラス基板１１の表面のプローブ生体分子を付着させたい特定の部位１０１のみにアビジン分子が単層に固定された固相化膜１４を形成することにより表面処理基板１００を得る。特定の部位１０１の面積及び形状は全て均一であるため、各特定の部位１０１に固定されたビオチン分子の数もほぼ均一である。したがって各特定の部位１０１に結合するアビジン分子の数は同一になる。この表面処理基板１００の各特定の部位１０１に、ビオチン化処理したプローブＤＮＡを含む溶液をスポットすることにより、ＤＮＡマイクロアレイを得る。ＤＮＡマイクロアレイは、各特定の部位１０１に固定されているアビジン分子の数が同一であるので、各特定の部位１０１に結合するプローブＤＮＡ２１の数も同一である。



【特許請求の範囲】

【請求項１】　プローブ生体分子を含む溶液を基板表面にスポットすることにより、当該溶液中のプローブ生体分子が基板表面の特定の部位のみに受容され固定化されるように表面処理してなる基板であって、
前記基板表面のほぼ全面にわたり、前記プローブ生体分子を各々定量的に受容し得る複数の微細なプローブ生体分子受容固相部を規則的に設けたことを特徴とする生体分子マイクロアレイ用基板。

【請求項２】　前記プローブ生体分子受容固相部は、アビジン、ストレプトアビジン、ビオチン、アミノ基、カルボニル基、水酸基、スクシニイド基、マレイド基、チオール基のうちのいずれかの固相化剤からなることを特徴とする請求項１に記載の生体分子マイクロアレイ用基板。

【請求項３】　前記基板は、ガラス基板、シリコン基板、プラスチック基板、金基板、銀基板のうちのいずれかであることを特徴とする請求項１または２に記載の生体分子マイクロアレイ用基板。

【請求項４】　前記プローブ生体分子受容固相部は、基板表面に結合したビオチン分子の末端にアビジン分子が単層に結合したものであることを特徴とする請求項１～３のいずれかに記載の生体分子マイクロアレイ用基板。

【請求項５】　請求項１～４のいずれかに記載の基板の前記プローブ生体分子受容固相部にプローブ生体分子が結合していることを特徴とする生体分子マイクロアレイ。

【請求項６】　前記プローブ生体分子は、ＤＮＡ、ＲＮＡ、ＰＮＡまたはタンパク質であることを特徴とする請求項５に記載の生体分子マイクロアレイ。

【請求項７】　前記プローブ生体分子はビオチンを標識した生体分子であり、前記プローブ生体分子受容固相部にビオチン－アビジン結合により結合していることを特徴とする請求項５または６に記載の生体分子マイクロアレイ。

【請求項８】　請求項１～４のいずれかに記載の生体分子マイクロアレイ用基板を製造するための方法であって、
フォトリソグラフィ技術およびエッチング技術を用いて、特定の部位のみ前記プローブ生体分子受容固相部を設ける工程を含むことを特徴とする生体分子マイクロアレイ用基板の製造方法。

【発明の詳細な説明】

【０００１】

【発明の属する技術分野】本願発明は、検出すべきターゲット生体分子に対して相補的な塩基配列を有する一本鎖の生体分子をプローブとし、当該プローブ生体分子と生体由来の試料核酸とのハイブリダイゼーションにより形成される二本鎖の有無を検出することによってターゲット生体分子を検出する生体分子検出技術に属し、特に、プローブ生体分子を含む溶液を基板の表面にスポットすることにより当該基板上に生体分子検出スポット部を形成してなる生体分子マイクロアレイに関するものである。

【０００２】

【従来の技術】生体由来の試料中に存在する生体分子（ＤＮＡ、ＲＮＡなど）を検出するためのデバイスとしてＤＮＡマイクロアレイ（ＤＮＡチップともよばれる）がある。ＤＮＡマイクロアレイによれば、数百～数万回分の生体分子検出処理もしくは塩基配列決定処理を一括して並列的に行うことが可能である。ＤＮＡマイクロアレイは、数平方センチメートル～十数平方センチメートルのガラス基板やシリコン基板上に数百～数万のＤＮＡ検出ポイント（スポット部）を整然と配置してなる。それぞれのＤＮＡ検出ポイントには予め既知の塩基配列を持った一本鎖の核酸ポリマー（遺伝子断片）がプローブ（検出子）として一種類ずつ固定されている。つまり、ＤＮＡマイクロアレイ上にはたくさんの種類の核酸プローブが整列している。このＤＮＡマイクロアレイ上に、蛍光物質でラベリング（標識）した試料核酸の水溶液を流すと、試料核酸中の核酸ポリマーの塩基配列がプローブと相補的である場合のみ両者がハイブリダイズし、洗浄後も、ＤＮＡマイクロアレイ上にプローブとハイブリダイズしたターゲット核酸ポリマーだけが残存する。ＤＮＡマイクロアレイ上に残存したターゲット核酸ポリマー中の蛍光物質が発する蛍光を検出することにより、試料核酸中にターゲット核酸ポリマーが存在するか否かを判定できる。

【０００３】ＤＮＡマイクロアレイは、製造法によってフォトリソグラフィ型とスポッティング型の２種類に大別できる。フォトリソグラフィ型では、半導体集積回路の製造プロセスで用いられるフォトリソグラフィによって基板（あるいはシート）上で所望の多種類のＤＮＡ（オリゴヌクレオチド）を合成する製造方法がとられ、高密度のＤＮＡ検出ポイントを有するＤＮＡマイクロアレイが実用化されている（米国特許５７４４３０５、５４４５９３４等参照）。一方、スポッティング型では、固相化剤（ポリリジンまたはアミノシラン）をスライドガラスの全面にコーティングした基板（あるいはシート）を用い、その基板上に、あらかじめ調製したプローブＤＮＡを含む水滴を一つ一つスポットして載せた後、乾燥させることにより、ＤＮＡ検出スポットを形成する製造方法がとられる（米国特許５８７５２２等参照）。

【０００４】

【発明が解決しようとする課題】上述した２種類のＤＮＡマイクロアレイには、以下のような特性の違いがある。フォトリソグラフィ型のＤＮＡマイクロアレイは、ＤＮＡ検出ポイントを細かくでき、ＤＮＡを均一に生やすことができるため、高い測定感度とその再現性を保証できる点、ＳＮＰ（一塩基多型）分析に使用できる点で

優れている。しかしながら、マスクは１塩基合成するために１枚必要であり、塩基はA、T、G、Cと４種類あるので、少なくとも４枚のマスクが必要となる。たとえば２０塩基の長さのプローブを合成するには８０枚のマスクが必要である。マスクは１枚数十万円と高価であり、DNAマイクロアレイを作るためには数千万円の費用がかかる。このため、一部の研究機関でしか使用されていないのが現状である。

【０００５】スポッティング型のDNAマイクロアレイは、プローブDNAを含む水滴を基板上に載せて乾かす方法を用いるため、基板上に固定されるDNAの密度と均一さが保証されない。すなわち、DNA検出スポット部の寸法や形状が不均一になるため、各DNA検出スポット部に固定されているDNA量にばらつきが生じる。このためスポッティング型のDNAマイクロアレイは、定性的な解析には使用できても、定量的な解析には向いていなかった。すなわち、ターゲット生体分子とのハイブリダイゼーションが生じたDNA検出スポット部の有無は検出できても、各DNA検出スポット部でハイブリダイゼーションしたターゲット生体分子の量を測定することはできなかった。また、DNA検出スポット部の周囲に付着した固相化剤の存在により、ターゲットDNAが非特異的に基板上に吸着し、ノイズの上昇を引き起こし、S／N比を低下させていた。本願発明は、このような事情の下に創案されたものであり、その目的は、定量的な解析に使用でき且つS／N比の高いスポッティング型の生体分子マイクロアレイを提供することにある。

【０００６】

【課題を解決するための手段】上記目的を達成するために、本願発明では以下の手段を採用する。本発明に係る生体分子マイクロアレイ用基板は、基板表面のほぼ全面にわたり、前記プローブ生体分子を各々定量的に受容し得る複数の微細なプローブ生体分子受容固相部を規則的に設けたことを特徴とする。本発明の生体分子マイクロアレイ用基板において、前記プローブ生体分子受容固相部は、アビジン、ストレプトアビジン、ビオチン、アミノ基、カルボニル基、水酸基、スクシニイド基、マレイド基、チオール基のうちのいずれかの固相化剤からなる。また、前記基板は、ガラス基板、シリコン基板、プラスチック基板、金基板、銀基板のうちのいずれかである。また、前記プローブ生体分子受容固相部は、基板表面に結合したビオチン分子の末端にアビジン分子が単層に結合したものである。また、前記特定の部位の径は５０～２００ミクロン、前記特定の部位同士の間隔は１００～５００ミクロンであることが望ましい。ここで、前記特定の部位の径とは、当該特定の部位の形状が円形の場合は直径、正方形の場合は一片の長さを意味する。また、前記特定の部位の形状が、前記生体分子マイクロアレイの生体分子検出スポット部の撮像に使用する固体撮像素子の画素の形状と略一致していることが望ましい。本発明に係る生体分子マイクロアレイは、請求項１～４のいずれかに記載の基板の前記プローブ生体分子受容固相部にプローブ生体分子を結合させたものであることを特徴とする。本発明の生体分子マイクロアレイにおいて、前記プローブ生体分子は、DNA、RNA、PNAまたはタンパク質である。また、前記プローブ生体分子はビオチンを標識した生体分子であり、前記プローブ生体分子受容固相部にビオチン－アビジン結合により結合している。本発明に係る製造方法は、前記生体分子マイクロアレイ用基板を製造する方法であって、フォトリソグラフィ技術およびエッチング技術を用いて、特定の部位のみ前記プローブ生体分子受容固相部を設ける工程を含むことを特徴とする。

【０００７】

【発明の実施の形態】以下、本発明の実施の形態について図面を参照して詳細に説明する。まず、本発明に係るDNAマイクロアレイ用基板（以下、単に表面処理基板と記す。）について説明する。図１は本発明に係る表面処理基板の製造方法の一例を示す製造工程図である。図中、１００は本発明に係る表面処理基板であり、この表面処理基板１００は、プローブDNAが特定の部位１０１のみに付着するように表面処理してなる。表面処理基板１００の製造工程は以下のとおりである。

（１）基板洗浄工程：スライドグラス基板１１を洗浄し不純物を取り除く。

（２）アルミニウム膜蒸着工程：スライドグラス基板１１の表面に、アルミニウム膜１２を蒸着（コーティング）する。

（３）フォトレジストの塗布工程：アルミニウム膜１２の表面にネガ型のフォトレジストを塗布（コーティング）する。

（４）露光工程：フォトマスク１４を通して（３）の基板上の特定の部位１０１にのみ光（$h\nu$）を照射する。

（５）現像工程：（４）の基板上のフォトレジスト１３を現像する。この段階で特定の部位１０１のフォトレジスト１３が除去される。

（６）エッチング工程：（５）の基板上のアルミニウム膜をエッチングする。この段階で、特定の部位１０１のアルミニウム膜１２が除去される。

（７）レジスト除去工程：（６）の基板上のフォトレジスト１３をアセトンにより溶解し除去する。この段階で、スライドグラス基板１１の表面が特定の部位１０１のみ露出する。

（８）DNA固相化膜形成工程：（７）の基板上に、プローブDNAを吸着し固相化する固相化剤を塗布し、DNA固相化膜１５を形成する。この工程は、具体的には、アミノシラン処理による基板表面へのアミノ基導入工程と、ビオチンスクシニイドによる基板表面のアミノ基へのビオチン導入工程とからなる。

（９）DNA付着部位形成工程：（８）の基板上のアル

ミニウム膜１２を酸、アルカリまたはキレート剤により溶解させて除去する。この段階で、スライドグラス基板１１の表面の特定の部位１０１にのみＤＮＡ固相化膜１５が形成される。

（１０）アビジン結合工程：（９）の基板上にアビジン溶液を導入し、特定の部位１０１に形成されたＤＮＡ固相化膜１５のビオチン分子の末端にアビジン分子を単層に結合させる。

以上の（１）～（１０）の工程を経ることにより、スライドグラス基板１１の表面の特定の部位１０１のみにアビジンが単層に固定されたＤＮＡマイクロアレイ基板１００が得られる。特定の部位１０１の直径は２００ミクロン以下、特定の部位１０１同士の間隔は４００ミクロン以下である。

【０００８】図２に各ＤＮＡ固相化膜１５のビオチン分子の末端にアビジン分子が単層に結合する過程を示す。各基板の表面に形成される特定の部位１０１の面積及び形状は全て均一であるため、各特定の部位１０１に固定されたビオチン分子２３の数もほぼ均一である。したがって、各特定の部位１０１に結合するアビジン分子の数は均等になる。すなわち、各特定の部位１０１に固定されたビオチンの数に多少ばらつきがあっても、アビジン分子の方がビオチン分子よりも遙かに大きいため、各特定の部位１０１に固定されるアビジン分子の数は一定になる。特定の部位１０１の形状や寸法、特定の部位１０１同士の間隔は、露光工程で使用するフォトマスクを変えることによって任意に変更できる。したがって、特定の部位１０１に固定するアビジン分子の数も、フォトマスクを変えることによって任意に制御できる。

【０００９】次に、本発明に係るＤＮＡマイクロアレイについて説明する。本発明に係るＤＮＡマイクロアレイは、図１の方法で製造された表面処理基板１００の各特定の部位１０１に、各々塩基配列の異なるプローブＤＮＡを含む溶液をスポットすることにより製造される。図３に本発明に係るＤＮＡマイクロアレイの製造方法の一例を示す。図中、１１０はプローブＤＮＡ２１を含む溶液１１１をＤＮＡマイクロアレイ基板１００上にスポットするためのアレイヤである。プローブＤＮＡ２１には、予めビオチンを標識したＤＮＡ（ビオチン化ＤＮＡ）を使用する。アレイヤ１１０は、毛細管作用により溶液１１１を一定量保持できるようになっており、溶液１１１を保持したアレイヤ１１０の先端を表面処理基板１００上の特定の部位１０１に突き当てることにより、一定量の溶液１１１が特定の部位１０１に供給される。その結果、溶液１１１中のプローブＤＮＡ２１がＤＮＡマイクロアレイ基板１００上の特定の部位１０１に固定されている各アビジン分子２２に１つずつ結合する（ビオチン化ＤＮＡの固相化）。各特定の部位１０１に固定されているアビジン分子２２の数は同一であるので、各特定の部位１０１に結合するプローブＤＮＡ２１の数も同一である（図４参照）。各特定の部位１０１に固定するアビジン分子の数は、上述したように、露光工程で使用するフォトマスクを変えて特定の部位１０１の形状や寸法を変更することにより任意に変更できる。したがって、各特定の部位１０１に固定するプローブＤＮＡ２１の数も、露光工程で使用するフォトマスクの変更により任意に制御でき、各特定の部位１０１にスポットする溶液１１１のプローブＤＮＡ濃度にばらつきがあっても、常に一定数のプローブＤＮＡ２１を各特定の部位１０１に固定することができる。

【００１０】図５に表面処理基板上の特定の部位にスポットする溶液中のＤＮＡ量（濃度）と特定の部位に固定化されるＤＮＡ量との関係の測定例を示す。この測定結果から、3×10^{9}～5×10^{9}個のプローブＤＮＡをスポットすれば特定の部位に固定化されるプローブＤＮＡ量が一定になることがわかる。ＤＮＡマイクロアレイ上のＤＮＡ検出スポット部は、全て同一形状、同一面積であり、しかも全検出スポット部に同数ずつプローブＤＮＡ２１が固定されているので、このＤＮＡマイクロアレイによれば定量的な解析が可能になる。ＤＮＡマイクロアレイ上におけるＤＮＡ吸着部位が特定の部位１０１すなわちＤＮＡ検出スポット部のみに制限されるので、ＤＮＡ検出スポット部の周囲にＤＮＡが非特異的は吸着を起こすのを防止できる。したがって、蛍光検出時のノイズ（不要光）の減少により、Ｓ／Ｎ比を向上させることができる。さらに、ＤＮＡ検出スポット部の形状すなわち前記特定の部位の形状を、撮像に使用する固体撮像素子（ＣＣＤセンサ、ＣＭＯＳセンサなど）の画素の形状と一致させておくことにより、Ｓ／Ｎ比をより向上させることができる。

【００１１】なお、本発明は以上の実施の形態に限定されるものではない。たとえば、本発明に係る表面処理基板の製造方法は上記の実施の形態に限定されない。すなわち、図１に示した製造方法では、基板１１の表面に、先ずアルミニウム膜１２、ポジ型のフォトレジスト１３を順次積層する。次に特定の部位１０１を規則的に配列してあるフォトマスクを通して、フォトレジストの特定の部位のみ露光し、現像液に浸すことで、特定の部位１０１のみフォトレジストが溶解し、特定の部位１０１のみアルミニウム膜１２が露出する。その後、アルミニウム膜１２を酸性のエッチング溶液によりエッチングすることにより、特定の部位１０１のアルミニウム膜１２が溶解され、基板１１の特定の部位１０１のガラス表面が露出する。その上にＤＮＡ固相化膜１５を塗布し、アルミニウム膜１２を溶解することにより、基板１１の表面の特定の部位１０１にのみＤＮＡ固相化膜１５を残すことができる。別な方法として、基板１１の表面全体に最初からＤＮＡ固相化膜１５を形成し、特定の部位１０１のみＤＮＡ固相化膜１５を露出させる方法を採用してもよい。この場合には、基板１１の表面全体にＤＮＡ固相

化膜１５およびアルミニウム膜１２を順次積層形成した後、アルミニウム膜１２上にポジ型のフォトレジスト１３を積層形成し、フォトマスクを通して特定の部位１０１にのみ露光し、上記と同じように、現像とエッチングとを行うことにより、特定の部位１０１のみＤＮＡ固相化膜１５を露出させることができる。また、フォトレジストはポジ型である必要はなく、ネガ型のフォトレジストも使用可能であることは無論である。

【００１２】また、上記実施の形態では、ＤＮＡマイクロアレイ、すなわちプローブ生体分子としてＤＮＡを固定した生体分子マイクロアレイについて説明したが、プローブ生体分子としてＲＮＡ、ＰＮＡ、蛋白質などを用いたものも本発明の生体分子マイクロアレイに含まれる。また、表面処理基板に用いる基板は、スライドグラス基板に限るものではなく、透明ガラス基板、シリコン基板、プラスチック基板、金基板、銀基板などでもよい。また、固相化剤に付いても、アビジンを固定（露出）したものに限らず、前記特定の部位に固定化したいプローブ生体分子との結合性を考慮して、固定化するプローブ生体分子数の定量化に最も適切と思われる物質を使用すればよい。

【００１３】

【発明の効果】以上説明したように、本発明は以下のような優れた効果を奏する。本発明に係る生体分子マイクロアレイ用基板は、プローブ生体分子が基板表面の特定の部位のみに付着するように表面処理されているので、プローブ生体分子を含む溶液を基板表面にスポットすることにより、生体分子検出スポット部の外の領域にプローブ生体分子が付着するのを防止して、Ｓ／Ｎ比の高い生体分子マイクロアレイを得ることができる。また、前記特定の部位の面積及び形状を変えることにより、生体分子検出スポット部に固定化するプローブ生体分子の量を制御することができる。前記特定の部位の面積及び形状を全て同一とすれば、全生体分子検出スポット部のプローブ生体分子の量を一定にすることができ、本発明に係る生体分子マイクロアレイが得られる。本発明に係る生体分子マイクロアレイによれば、各生体分子検出スポット部に固定化されているプローブ生体分子の量が一定であるので、ターゲット生体分子の定量的な解析に使用できる。また、プローブ生体分子を特定の部位に固定化することで生体分子検出スポット部が形成されているので、生体分子検出スポット部の外の領域におけるターゲット生体分子の非特異的吸着を防止し、Ｓ／Ｎ比の高い測定を行うことができる。本発明に係る製造方法によれば、プローブ生体分子を吸着し固相化する固相化膜をフォトリソグラフィなどの精密加工技術を用いて基板表面の特定の部位のみに形成することにより、本発明に係る生体分子マイクロアレイ用表面処理基板を高精度に製造することができる。

【図面の簡単な説明】

【図１】本発明に係る表面処理基板の製造方法の一例を示す製造工程図である。

【図２】各ＤＮＡ固相化膜のビオチン分子の末端にアビジン分子が結合する過程に関する説明図である。

【図３】本発明に係るＤＮＡマイクロアレイの製造方法の一例を示す説明図である。

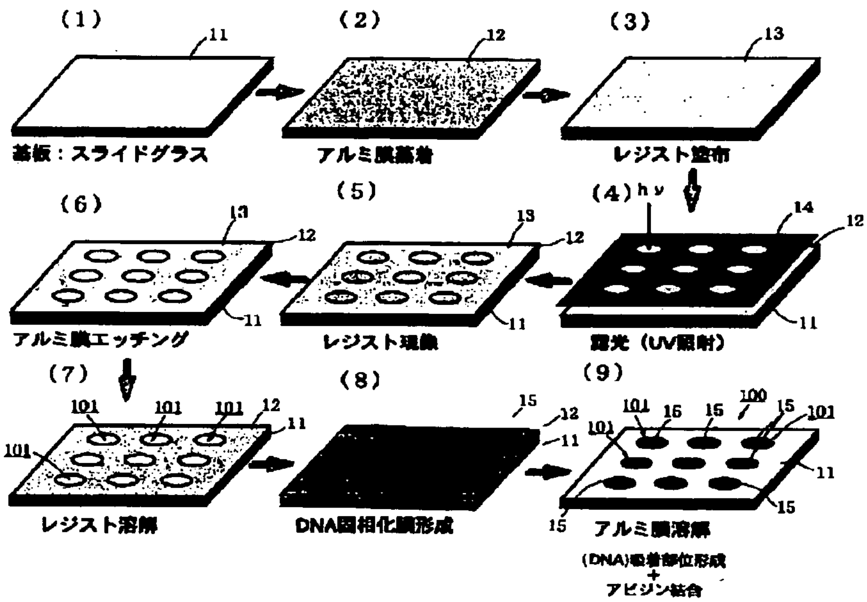
【図４】特定の部位に固定されているアビジン分子にプローブＤＮＡ（ビオチン化ＤＮＡ）が結合する過程に関する説明図である。

【図５】表面処理基板上の特定の部位にスポットする溶液中のＤＮＡ量（濃度）と特定の部位に固定化されるＤＮＡ量との関係の測定結果を示す図である。

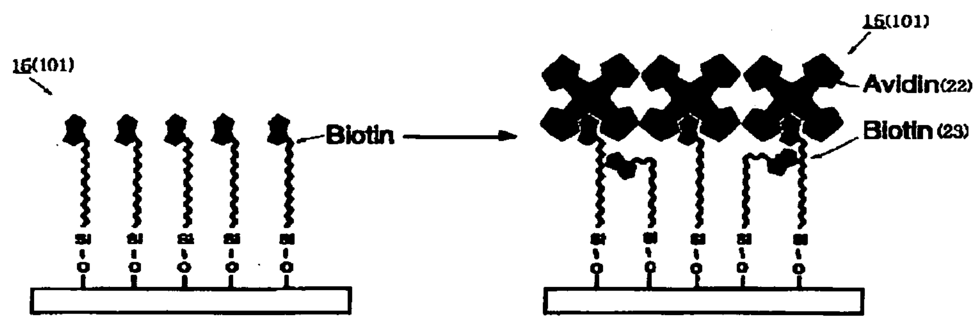
【符号の説明】

１１：スライドグラス基板
１２：アルミニウム膜
１３：フォトレジスト
１４：フォトマスク
１５：固相化膜
２１：プローブＤＮＡ
２２：アビジン分子
２３：ビオチン分子
１００：ＤＮＡマイクロアレイ表面処理基板
１０１：特定の部位
１１０：アレイヤ
１１１：溶液

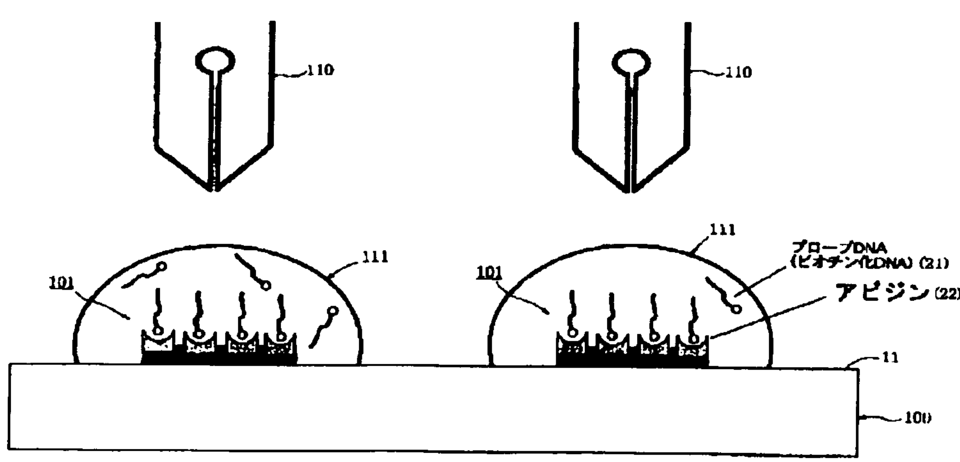
【図１】


(1)
(2)
(3)
基板：スライドグラス
アルミ膜蒸着
レジスト塗布
(4) hν
(5)
(6)
露光（UV照射）
レジスト現像
アルミ膜エッチング
(7)
(8)
(9)
レジスト溶解
DNA固相化膜形成
アルミ膜溶解
(DNA)吸着部位形成
+
アビジン結合

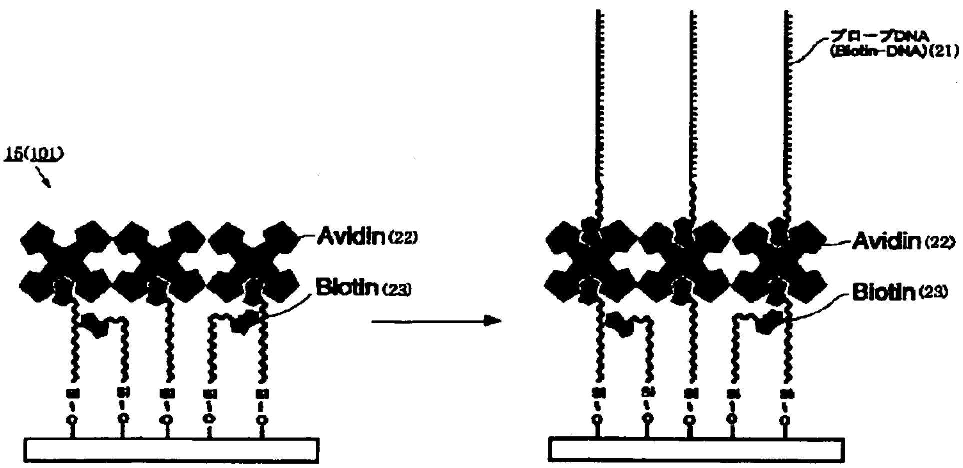
【図２】


15(101)
Biotin
15(101)
Avidin(22)
Biotin(23)

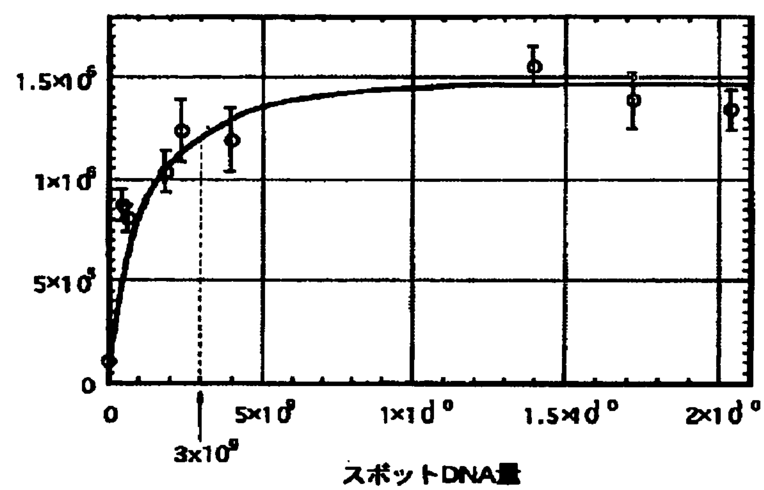
【図３】


プローブDNA
(ビオチン化DNA) (21)
アビジン(22)

【図４】



【図５】



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(72)発明者　近藤　恭光
埼玉県和光市広沢２番１号　理化学研究所内

(72)発明者　橘内　徳司
埼玉県和光市広沢２番１号　理化学研究所内

(72)発明者　竹中　繁織
福岡県古賀市舞の里４丁目23－21

(72)発明者　松本　和子
東京都新宿区戸塚町１丁目104番地　学校法人早稲田大学内

(72)発明者　野島　高彦
東京都新宿区戸塚町１丁目104番地　学校法人早稲田大学内

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(71)Applicant : INST OF PHYSICAL & CHEMICAL RES
UNIV WASEDA

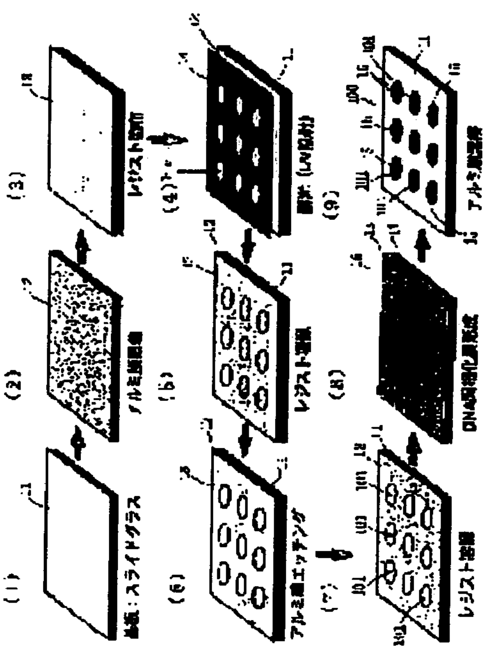
(72)Inventor : TASHIRO HIDEO
KONDO YASUMITSU
KITSUNAI TOKUJI
TAKENAKA SHIGEORI
MATSUMOTO KAZUKO
NOJIMA TAKAHIKO

(54) BIOMOLECULE MICROARRAY

(57)Abstract:

PROBLEM TO BE SOLVED: To provide a biomolecule microarray which can be used for quantitative analyses and has a high S/N ratio.

SOLUTION: A photolithography technique and an etching technique are used to form an immobilizing film 14 in which avidin molecules are immobilized in a single layer at only specific probe biomolecule adhesion-desired sites 101 on the surface of a slide glass substrate 11, thus obtaining the surface-treated substrate 100. Since the areas and shapes of the specific sites all are uniform, the numbers of the biotin molecules immobilized on the specific sites 101 are also approximately uniform. Thereby, the numbers of the avidin molecoles bound to the specific sites 101 are identical. The specific sites 101 of the surface- treated substrate 100 are spotted with a solution containing probe DNA treated with biotin to obtain the DNA microarray. Since the numbers of the avidin molecules immobilized on the specific sites 101 are identical in the DNA microarrays, the number of probe DNAs 21 bound to the specific sites 101 is also identical.



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[Kind of final disposal of application other than the examiner's decision of rejection or application converted registration]

[Date of final disposal for application]

[Patent number]

[Date of registration]

[Number of appeal against examiner's decision of rejection]

[Date of requesting appeal against examiner's decision of rejection]

[Date of extinction of right]

* NOTICES *

CLAIMS

[Claim(s)]
[Claim 1] The substrate for biomolecule microarrays which is a substrate which carries out surface preparation and becomes as the probe biomolecule in the solution concerned is received and fixed by only the specific part on the front face of a substrate by carrying out the spot of the solution containing probe biomolecule to a substrate front face, and is characterized by to prepare regularly two or more detailed probe biomolecule acceptance solid phase sections on said front face of a substrate which migrate to the whole surface mostly and may receive said probe biomolecule quantitatively respectively.
[Claim 2] Said probe biomolecule acceptance solid phase section is a substrate for biomolecule microarrays according to claim 1 characterized by consisting of a solid phase-ized agent of the avidin, streptoavidin, a biotin, the amino group, a carbonyl group, a hydroxyl group, a SUKUSHI need radical, a MAREIDO radical, or the thiol groups.
[Claim 3] Said substrate is a substrate for biomolecule microarrays according to claim 1 or 2 characterized by being a glass substrate, a silicon substrate, a plastic plate, a golden substrate, or the silver substrates.
[Claim 4] Said probe biomolecule acceptance solid phase section is a substrate for biomolecule microarrays according to claim 1 to 3 characterized by an avidin molecule combining with a monolayer at the end of the biotin molecule combined with the substrate front face.
[Claim 5] The biomolecule microarray characterized by probe biomolecule having combined with said probe biomolecule acceptance solid phase section of a substrate according to claim 1 to 4.
[Claim 6] Said probe biomolecule is a biomolecule microarray according to claim 5 characterized by being DNA, RNA, PNA, or protein.
[Claim 7] Said probe biomolecule is a biomolecule microarray according to claim 5 or 6 which is the biomolecule which carried out the indicator of the biotin, and is characterized by having combined with said probe biomolecule acceptance solid phase section by biotin-avidin association.
[Claim 8] The manufacture approach of the substrate for biomolecule microarrays which is an approach for manufacturing the substrate for biomolecule microarrays according to claim 1 to 4, and is characterized by including the process in which only a specific part prepares said probe biomolecule acceptance solid phase section using a photolithography technique and an etching technique.

[Translation done.]

* NOTICES *

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1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] The invention in this application uses as a probe biomolecule of the single strand which has a complementary base sequence to the target biomolecule which should be detected, belongs to the biomolecule detection technique detect target biomolecule by detecting the existence of the double strand formed of the hybridization of the probe biomolecule concerned and the sample nucleic acid of the living body origin, and relates to the biomolecule microarray which comes to form the biomolecule detection spot section on the substrate concerned by carrying out the spot of the solution which contains probe biomolecule especially on the surface of a substrate.
[0002]
[Description of the Prior Art] There is a DNA microarray (called a DNA chip) as a device for detecting the biomolecules (DNA, RNA, etc.) which exist in the sample of the living body origin. According to the DNA microarray, it is possible for biomolecule detection processing or base-sequence-determination processing of hundreds - tens of thousands batches to be put in block, and to perform it in juxtaposition. A DNA microarray comes to arrange the DNA detection point (spot section) of hundreds - a-10,000 number on a several square centimeters - about ten square centimeters glass substrate or a silicon substrate tidily. One kind of nucleic-acid polymer (gene fragment) of a single strand which had a known base sequence beforehand is being fixed to each DNA detection point at a time as a probe (sensor). That is, on a DNA microarray, many kinds of nucleic acid probes have aligned. If the water solution of the sample nucleic acid which carried out labeling (indicator) with the fluorescent material on this DNA microarray is poured, only when the base sequence of the nucleic-acid polymer in a sample nucleic acid is as complementary as FUROBU, both will hybridize, and also even in after washing, only a probe and the target nucleic-acid polymer to which it hybridized remain on a DNA microarray. By detecting the fluorescence which the fluorescent material in the target nucleic-acid polymer which remained on the DNA microarray emits, it can judge whether a target nucleic-acid polymer exists in a sample nucleic acid.
[0003] A DNA microarray can be divided roughly into two kinds, a photolithography mold and a spotting mold, according to a manufacturing method. In the photolithography mold, the manufacture approach which compounds DNA (oligonucleotide) of desired varieties on a substrate (or sheet) by the photolithography used in the manufacture process of a semiconductor integrated circuit is taken, and the DNA microarray which has the DNA detection point of high density is put in practical use (refer to U.S. Pat. No. 5744305 and 5445934 grade). On the other hand, in a spotting mold, after carrying out the spots of the waterdrop containing the probe DNA which prepared beforehand the solid phase-ized agent (the poly lysine or amino silane) on the substrate using the substrate (or sheet) with which the whole surface of slide glass was coated one by one and carrying it, the manufacture approach which forms a DNA detection spot is taken by making it dry (reference, such as U.S. Pat. No. 587522).
[0004]
[Problem(s) to be Solved by the Invention] There is a difference among the following properties

in two kinds of DNA microarrays mentioned above. Since the DNA microarray of a photolithography mold can make the DNA detection point fine and can grow DNA to homogeneity, it is excellent in the point that high sensitometry and its repeatability can be guaranteed, and the point which can be used for SNP (a little salt radical polymorphism) analysis. However, one mask is required in order to compound one base, and since there are four kinds of bases with A, T, G, and C, at least four masks are needed. For example, 80 masks are required to compound the probe of the die length of 20 bases. The mask is as expensive as hundreds of thousands of yen per sheet, and in order to make a DNA microarray, it requires tens of millions of yen costs. For this reason, the present condition is used only by some research facilities.
[0005] In order to use for the DNA microarray of a spotting mold the approach of carrying the waterdrop containing probe DNA on a substrate, and drying it, the consistency and uniformity of DNA which are fixed on a substrate are not guaranteed. That is, since the dimension and configuration of the DNA detection spot section become an ununiformity, dispersion arises in the amount of DNA currently fixed to each DNA detection spot section. For this reason, even if the DNA microarray of a spotting mold was applicable to qualitative analysis, it was not fit for quantitive analysis. That is, even if the existence of the DNA detection spot section which the hybridization with target biomolecule produced was detectable, it was not able to measure the amount of the target biomolecule which carried out hybridization in each DNA detection spot section. Moreover, by existence of the solid phase-ized agent adhering to the perimeter of the DNA detection spot section, Target DNA adsorbed on the substrate nonspecific, the rise of a noise was caused, and the S/N ratio was reduced. It is originated under such a situation and the invention in this application has the purpose in being able to use it for quantitive analysis and offering the biomolecule microarray of the high spotting mold of a S/N ratio.
[0006]
[Means for Solving the Problem] In order to attain the above-mentioned purpose, the following means are adopted in the invention in this application. The substrate for biomolecule microarrays concerning this invention is characterized by preparing regularly the detailed probe biomolecule acceptance solid phase section of the plurality on the front face of a substrate which migrates to the whole surface mostly and may receive said probe biomolecule quantitatively respectively. In the substrate for biomolecule microarrays of this invention, said probe biomolecule acceptance solid phase section consists of a solid phase-ized agent of the avidin, streptoavidin, a biotin, the amino group, a carbonyl group, a hydroxyl group, a SUKUSHI need radical, a MAREIDO radical, or the thiol groups. Moreover, said substrates are a glass substrate, a silicon substrate, a plastic plate, a golden substrate, or the silver substrates. Moreover, an avidin molecule combines said probe biomolecule acceptance solid phase section with the end of the biotin molecule combined with the substrate front face at a monolayer. Moreover, as for spacing of 50-200 microns and said specific parts, it is [the path of said specific part] desirable that it is 100-500 microns. Here, as for the case of a diameter and a square, the path of said specific part means the die length of a piece, when the configuration of the specific part concerned is circular. Moreover, it is desirable for the configuration of said specific part to carry out abbreviation coincidence with the configuration of the pixel of the solid state image sensor used for the image pick-up of the biomolecule detection spot section of said biomolecule microarray. The biomolecule microarray concerning this invention is characterized by combining probe biomolecule with said probe biomolecule acceptance solid phase section of a substrate according to claim 1 to 4. In the biomolecule microarray of this invention, said probe biomolecule is DNA, RNA, PNA, or protein. Moreover, said probe biomolecule is the biomolecule which carried out the indicator of the biotin, and is combined with said probe biomolecule acceptance solid phase section by biotin-avidin association. The manufacture approach concerning this invention is an approach of manufacturing said substrate for biomolecule microarrays, and is characterized by including the process in which only a specific part prepares said probe biomolecule acceptance solid phase section using a photolithography technique and an etching technique.
[0007]
[Embodiment of the Invention] Hereafter, the gestalt of operation of this invention is explained to a detail with reference to a drawing. First, the substrate for DNA microarrays concerning this

invention (it is only hereafter described as a surface treatment substrate.) is explained. Drawing 1 is the production process Fig. showing an example of the manufacture approach of the surface treatment substrate concerning this invention. Among drawing, 100 are a surface-preparation substrate concerning this invention, surface preparation is carried out and this surface-preparation substrate 100 becomes so that probe DNA may adhere only to the specific part 101. The production process of the surface treatment substrate 100 is as follows.
(1) Substrate washing process : wash the slide glass substrate 11 and remove an impurity.
(2) Aluminum film vacuum evaporationo process : vapor-deposit the aluminum film 12 on the front face of the slide glass substrate 11 (coating).
(3) The spreading process of a photoresist : apply the photoresist of a negative mold to the front face of the aluminum film 12 (coating).
(4) Exposure process : irradiate light (hnu) only to the specific part 101 on the substrate of (3) through a photo mask 14.
(5) Develop the photoresist 13 on the substrate of development process: (4). The photoresist 13 of the specific part 101 is removed in this phase.
(6) Etch the aluminum film on the substrate of etching process: (5). In this phase, the aluminum film 12 of the specific part 101 is removed.
(7) Dissolve with an acetone and remove the photoresist 13 on the substrate of resist removal process: (6). In this phase, the front face of the slide glass substrate 11 exposes only the specific part 101.
(8) On the substrate of DNA solid phase-ized film formation process: (7), apply the solid phase-ized agent which adsorbs probe DNA and solid-phase-izes it, and form the DNA solid phase-ized film 15. Specifically, this process consists of an amino-group installation process on the front face of a substrate by amino silanizing, and a biotin installation process to the amino group on the front face of a substrate by the biotin SUKUSHI need.
(9) Make it dissolve by the acid, alkali, or the chelating agent, and remove the aluminum film 12 on the substrate of DNA attachment site formation process: (8). The DNA solid phase-ized film 15 is formed only in the specific part 101 of the front face of the slide glass substrate 11 in this phase.
(10) Introduce an avidin solution on the substrate of avidin joint process: (9), and make a monolayer combine an avidin molecule with the end of the biotin molecule of the DNA solid phase-ized film 15 formed in the specific part 101.
By passing through the process of the above (1) - (10), the DNA microarray substrate 100 with which avidin was fixed only to the specific part 101 of the front face of the slide glass substrate 11 by the monolayer is obtained. Spacing of 200 microns or less and part 101 specific comrades of the diameter of the specific part 101 is 400 microns or less.
[0008] The process which an avidin molecule combines with a monolayer is shown in the end of the biotin molecule of each DNA solid phase-ized film 15 at drawing 2 . Since all of a specific area and the specific configuration of a part 101 which are formed in the front face of each substrate are homogeneity, its number of the biotin molecules 23 fixed to each specific part 101 is also almost uniform. Therefore, the number of the avidin molecules combined with each specific part 101 becomes equal. That is, even if some dispersion is in the number of the biotins fixed to each specific part 101, the number of the avidin molecules with which the direction of an avidin molecule is fixed to each specific part 101 since it is larger than a biotin molecule for whether your being Haruka becomes fixed. Spacing of the configuration of the specific part 101, a dimension, and part 101 specific comrades can be changed into arbitration by changing the photo mask used at an exposure process. Therefore, the number of the avidin molecules fixed to the specific part 101 is also controllable to arbitration by changing a photo mask.
[0009] Next, the DNA microarray concerning this invention is explained. The DNA microarray concerning this invention is manufactured by each specific part 101 of the surface-preparation substrate 100 manufactured by the approach of drawing 1 by carrying out the spot of the solution containing the probe DNA from which a base sequence differs respectively. An example of the manufacture approach of the DNA microarray which starts this invention at drawing 3 is shown. 110 are AREIYA for carrying out the spot of the solution 111 containing probe DNA 21 on

the DNA microarray substrate 100 among drawing. DNA (biotin-ized DNA) which carried out the indicator of the biotin beforehand is used for probe DNA 21. AREIYA 110 is supplied to the part 101 of specification [the solution 111 of a constant rate] by dashing the tip of AREIYA 110 which it had come to be able to carry out the constant-rate maintenance of the solution 111 by capillarity, and held the solution 111 against the specific part 101 on the surface treatment substrate 100. Consequently, the probe DNA 21 in a solution 111 combines with each one avidin molecule 22 of every currently fixed to the specific part 101 on the DNA microarray substrate 100 (solid-phase-izing of biotin-ized DNA). Since the number of the avidin molecules 22 currently fixed to each specific part 101 is the same, the number of the probe DNA 21 combined with each specific part 101 is also the same (refer to drawing 4). The number of the avidin molecules fixed to each specific part 101 can be changed into arbitration by changing the photo mask used at an exposure process, and changing the specific configuration and specific dimension of a part 101, as mentioned above. Therefore, it is controllable by modification of the photo mask used at an exposure process to arbitration, and the number of the probe DNA 21 fixed to each specific part 101 can also always fix a fixed number of probe DNA 21 to each specific part 101, even if dispersion is in the probe DNA concentration of the solution 111 which carries out a spot to each specific part 101.
[0010] The example of measurement of the relation between the amount of DNA in the solution which carries out a spot to drawing 5 to the specific part on a surface treatment substrate (concentration), and the amount of DNA fixed by the specific part is shown. This measurement result shows that the amount of probe DNA fixed by the specific part becomes fixed, if the spot of the 3x109 to 5x109 probe DNA is carried out. All the DNA detection spot sections on a DNA microarray are the same configuration and the same area, and since same number [every] probe DNA 21 is moreover being fixed to all the detection spot sections, according to this DNA microarray, quantitive analysis is attained. Since the DNA adsorption site on a DNA microarray is restricted to the specific part 101, i.e., the DNA detection spot section, DNA can prevent that an un-unique target causes adsorption around the DNA detection spot section. Therefore, a S/N ratio can be raised by reduction of the noise at the time of fluorescence detection (unnecessary light). Furthermore, a S/N ratio can be raised more by making it in agreement with the configuration of the pixel of the solid state image sensors (a CCD sensor, CMOS sensor, etc.) which use the configuration of the DNA detection spot section, i.e., the configuration of said specific part, for an image pick-up.
[0011] In addition, this invention is not limited to the gestalt of the above operation. For example, the manufacture approach of the surface treatment substrate concerning this invention is not limited to the gestalt of the above-mentioned operation. That is, by the manufacture approach shown in drawing 1 , the laminating of the photoresist 13 of the aluminum film 12 and a positive type is first carried out to the front face of a substrate 11 one by one. Next, it lets the photo mask which has arranged the specific part 101 regularly pass, and only the specific part of a photoresist is exposed, by dipping in a developer, a photoresist dissolves only the specific part 101 and the aluminum film 12 exposes only the specific part 101. Then, by etching the aluminum film 12 with an acid etching solution, the aluminum film 12 of the specific part 101 is dissolved, and the glass front face of the specific part 101 of a substrate 11 is exposed. It can leave the DNA solid phase-ized film 15 only to the specific part 101 of the front face of a substrate 11 by applying the DNA solid phase-ized film 15 on it, and dissolving the aluminum film 12. As another approach, the DNA solid phase-ized film 15 may be formed in the whole front face of a substrate 11 from the beginning, and only the specific part 101 may adopt the approach of exposing the DNA solid phase-ized film 15. In this case, after carrying out laminating formation of the DNA solid phase-ized film 15 and the aluminum film 12 one by one on the whole front face of a substrate 11, only the specific part 101 can expose the DNA solid phase-ized film 15 by carrying out laminating formation of the photoresist 13 of a positive type on the aluminum film 12, exposing only to the specific part 101 through a photo mask, and performing development and etching like the above. Moreover, a photoresist does not need to be a positive type and it is undoubted that the photoresist of a negative mold is also usable.
[0012] Moreover, although the gestalt of the above-mentioned implementation explained the

DNA microarray, i.e., the biomolecule microarray which fixed DNA as probe biomolecule, the thing using RNA, PNA, protein, etc. as probe biomolecule is also contained in the biomolecule microarray of this invention. Moreover, the substrate used for a surface treatment substrate may not be restricted to a slide glass substrate, and a transparence glass substrate, a silicon substrate, a plastic plate, a golden substrate, a silver substrate, etc. are sufficient as it. Moreover, what is necessary is just to use the matter considered to be the most suitable for quantification of the number of probe biomolecules to fix in consideration of affinity with probe biomolecule to fix not only to what fixed avidin (exposure) but to said specific part, even if attached to a solid phase-ized agent.

[0013]

[Effect of the Invention] As explained above, this invention does the following outstanding effectiveness so. Since surface preparation of the substrate for biomolecule microarrays concerning this invention is carried out so that probe biomolecule may adhere only to the specific part on the front face of a substrate, by carrying out the spot of the solution containing probe biomolecule to a substrate front face, it can prevent that probe biomolecule adheres to the field besides the biomolecule detection spot section, and can obtain the high biomolecule microarray of a S/N ratio. Moreover, the amount of the probe biomolecule fixed in the biomolecule detection spot section is controllable by changing said specific area and specific configuration of a part. The same, then the biomolecule microarray which can make regularity the amount of the probe biomolecule of all the biomolecule detection spot sections, and starts this invention are obtained in all of said specific area and specific configuration of a part. Since the amount of the probe biomolecule fixed by each biomolecule detection spot section is fixed according to the biomolecule microarray concerning this invention, it can be used for the quantitive analysis of target biomolecule. Moreover, since the biomolecule detection spot section is formed by fixing probe biomolecule to a specific part, the nonspecific adsorption of the target biomolecule in the field besides the biomolecule detection spot section can be prevented, and high measurement of a S/N ratio can be performed. According to the manufacture approach concerning this invention, the surface treatment substrate for biomolecule microarrays concerning this invention can be manufactured with high precision by forming only in the specific part on the front face of a substrate the solid phase-ized film which adsorbs probe biomolecule and solid-phase-izes it using high precision processing technology, such as a photolithography.

[Translation done.]

TECHNICAL FIELD

[Field of the Invention] The invention in this application uses as a probe biomolecule of the single strand which has a complementary base sequence to the target biomolecule which should be detected, belongs to the biomolecule detection technique detect target biomolecule by detecting the existence of the double strand formed of the hybridization of the probe biomolecule concerned and the sample nucleic acid of the living body origin, and relates to the biomolecule microarray which comes to form the biomolecule detection spot section on the substrate concerned by carrying out the spot of the solution which contains probe biomolecule especially on the surface of a substrate.

[Translation done.]

PRIOR ART

[Description of the Prior Art] There is a DNA microarray (called a DNA chip) as a device for detecting the biomolecules (DNA, RNA, etc.) which exist in the sample of the living body origin. According to the DNA microarray, it is possible for biomolecule detection processing or base-sequence-determination processing of hundreds - tens of thousands batches to be put in block, and to perform it in juxtaposition. A DNA microarray comes to arrange the DNA detection point (spot section) of hundreds - a-10,000 number on a several square centimeters - about ten square centimeters glass substrate or a silicon substrate tidily. One kind of nucleic-acid polymer (gene fragment) of a single strand which had a known base sequence beforehand is being fixed to each DNA detection point at a time as a probe (sensor). That is, on a DNA microarray, many kinds of nucleic acid probes have aligned. If the water solution of the sample nucleic acid which carried out labeling (indicator) with the fluorescent material on this DNA microarray is poured, only when the base sequence of the nucleic-acid polymer in a sample nucleic acid is as complementary as FUROBU, both will hybridize, and also even in after washing, only a probe and the target nucleic-acid polymer to which it hybridized remain on a DNA microarray. By detecting the fluorescence which the fluorescent material in the target nucleic-acid polymer which remained on the DNA microarray emits, it can judge whether a target nucleic-acid polymer exists in a sample nucleic acid.

[0003] A DNA microarray can be divided roughly into two kinds, a photolithography mold and a spotting mold, according to a manufacturing method. In the photolithography mold, the manufacture approach which compounds DNA (oligonucleotide) of desired varieties on a substrate (or sheet) by the photolithography used in the manufacture process of a semiconductor integrated circuit is taken, and the DNA microarray which has the DNA detection point of high density is put in practical use (refer to U.S. Pat. No. 5744305 and 5445934 grade). On the other hand, in a spotting mold, after carrying out the spots of the waterdrop containing the probe DNA which prepared beforehand the solid phase-ized agent (the poly lysine or amino silane) on the substrate using the substrate (or sheet) with which the whole surface of slide glass was coated one by one and carrying it, the manufacture approach which forms a DNA detection spot is taken by making it dry (reference, such as U.S. Pat. No. 587522).

[Translation done.]

EFFECT OF THE INVENTION

[Effect of the Invention] As explained above, this invention does the following outstanding effectiveness so. Since surface preparation of the substrate for biomolecule microarrays concerning this invention is carried out so that probe biomolecule may adhere only to the specific part on the front face of a substrate, by carrying out the spot of the solution containing probe biomolecule to a substrate front face, it can prevent that probe biomolecule adheres to the field besides the biomolecule detection spot section, and can obtain the high biomolecule microarray of a S/N ratio. Moreover, the amount of the probe biomolecule fixed in the biomolecule detection spot section is controllable by changing said specific area and specific configuration of a part. The same, then the biomolecule microarray which can make regularity the amount of the probe biomolecule of all the biomolecule detection spot sections, and starts this invention are obtained in all of said specific area and specific configuration of a part. Since the amount of the probe biomolecule fixed by each biomolecule detection spot section is fixed according to the biomolecule microarray concerning this invention, it can be used for the quantitive analysis of target biomolecule. Moreover, since the biomolecule detection spot section is formed by fixing probe biomolecule to a specific part, the nonspecific adsorption of the target biomolecule in the field besides the biomolecule detection spot section can be prevented, and high measurement of a S/N ratio can be performed. According to the manufacture approach concerning this invention, the surface treatment substrate for biomolecule microarrays concerning this invention can be manufactured with high precision by forming only in the specific part on the front face of a substrate the solid phase-ized film which adsorbs probe biomolecule and solid-phase-izes it using high precision processing technology, such as a photolithography.

[Translation done.]

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] There is a difference among the following properties in two kinds of DNA microarrays mentioned above. Since the DNA microarray of a photolithography mold can make the DNA detection point fine and can grow DNA to homogeneity, it is excellent in the point that high sensitometry and its repeatability can be guaranteed, and the point which can be used for SNP (a little salt radical polymorphism) analysis. However, one mask is required in order to compound one base, and since there are four kinds of bases with A, T, G, and C, at least four masks are needed. For example, 80 masks are required to compound the probe of the die length of 20 bases. The mask is as expensive as hundreds of thousands of yen per sheet, and in order to make a DNA microarray, it requires tens of millions of yen costs. For this reason, the present condition is used only by some research facilities.
[0005] In order to use for the DNA microarray of a spotting mold the approach of carrying the waterdrop containing probe DNA on a substrate, and drying it, the consistency and uniformity of DNA which are fixed on a substrate are not guaranteed. That is, since the dimension and configuration of the DNA detection spot section become an ununiformity, dispersion arises in the amount of DNA currently fixed to each DNA detection spot section. For this reason, even if the DNA microarray of a spotting mold was applicable to qualitative analysis, it was not fit for quantitive analysis. That is, even if the existence of the DNA detection spot section which the hybridization with target biomolecule produced was detectable, it was not able to measure the amount of the target biomolecule which carried out hybridization in each DNA detection spot section. Moreover, by existence of the solid phase-ized agent adhering to the perimeter of the DNA detection spot section, Target DNA adsorbed on the substrate nonspecific, the rise of a noise was caused, and the S/N ratio was reduced. It is originated under such a situation and the invention in this application has the purpose in being able to use it for quantitive analysis and offering the biomolecule microarray of the high spotting mold of a S/N ratio.

[Translation done.]

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2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

MEANS

[Means for Solving the Problem] In order to attain the above-mentioned purpose, the following means are adopted in the invention in this application. The substrate for biomolecule microarrays concerning this invention is characterized by preparing regularly the detailed probe biomolecule acceptance solid phase section of the plurality on the front face of a substrate which migrates to the whole surface mostly and may receive said probe biomolecule quantitatively respectively. In the substrate for biomolecule microarrays of this invention, said probe biomolecule acceptance solid phase section consists of a solid phase-ized agent of the avidin, streptoavidin, a biotin, the amino group, a carbonyl group, a hydroxyl group, a SUKUSHI need radical, a MAREIDO radical, or the thiol groups. Moreover, said substrates are a glass substrate, a silicon substrate, a plastic plate, a golden substrate, or the silver substrates. Moreover, an avidin molecule combines said probe biomolecule acceptance solid phase section with the end of the biotin molecule combined with the substrate front face at a monolayer. Moreover, as for spacing of 50-200 microns and said specific parts, it is [the path of said specific part] desirable that it is 100-500 microns. Here, as for the case of a diameter and a square, the path of said specific part means the die length of a piece, when the configuration of the specific part concerned is circular. Moreover, it is desirable for the configuration of said specific part to carry out abbreviation coincidence with the configuration of the pixel of the solid state image sensor used for the image pick-up of the biomolecule detection spot section of said biomolecule microarray. The biomolecule microarray concerning this invention is characterized by combining probe biomolecule with said probe biomolecule acceptance solid phase section of a substrate according to claim 1 to 4. In the biomolecule microarray of this invention, said probe biomolecule is DNA, RNA, PNA, or protein. Moreover, said probe biomolecule is the biomolecule which carried out the indicator of the biotin, and is combined with said probe biomolecule acceptance solid phase section by biotin-avidin association. The manufacture approach concerning this invention is an approach of manufacturing said substrate for biomolecule microarrays, and is characterized by including the process in which only a specific part prepares said probe biomolecule acceptance solid phase section using a photolithography technique and an etching technique.
[0007]
[Embodiment of the Invention] Hereafter, the gestalt of operation of this invention is explained to a detail with reference to a drawing. First, the substrate for DNA microarrays concerning this invention (it is only hereafter described as a surface treatment substrate.) is explained. Drawing 1 is the production process Fig. showing an example of the manufacture approach of the surface treatment substrate concerning this invention. Among drawing, 100 are a surface-preparation substrate concerning this invention, surface preparation is carried out and this surface-preparation substrate 100 becomes so that probe DNA may adhere only to the specific part 101. The production process of the surface treatment substrate 100 is as follows.
(1) Substrate washing process : wash the slide glass substrate 11 and remove an impurity.
(2) Aluminum film vacuum evaporationo process : vapor-deposit the aluminum film 12 on the front face of the slide glass substrate 11 (coating).
(3) The spreading process of a photoresist : apply the photoresist of a negative mold to the front face of the aluminum film 12 (coating).

(4) Exposure process : irradiate light (hnu) only to the specific part 101 on the substrate of (3) through a photo mask 14.
(5) Develop the photoresist 13 on the substrate of development process: (4). The photoresist 13 of the specific part 101 is removed in this phase.
(6) Etch the aluminum film on the substrate of etching process: (5). In this phase, the aluminum film 12 of the specific part 101 is removed.
(7) Dissolve with an acetone and remove the photoresist 13 on the substrate of resist removal process: (6). In this phase, the front face of the slide glass substrate 11 exposes only the specific part 101.
(8) On the substrate of DNA solid phase-ized film formation process: (7), apply the solid phase-ized agent which adsorbs probe DNA and solid-phase-izes it, and form the DNA solid phase-ized film 15. Specifically, this process consists of an amino-group installation process on the front face of a substrate by amino silanizing, and a biotin installation process to the amino group on the front face of a substrate by the biotin SUKUSHI need.
(9) Make it dissolve by the acid, alkali, or the chelating agent, and remove the aluminum film 12 on the substrate of DNA attachment site formation process: (8). The DNA solid phase-ized film 15 is formed only in the specific part 101 of the front face of the slide glass substrate 11 in this phase.
(10) Introduce an avidin solution on the substrate of avidin joint process: (9), and make a monolayer combine an avidin molecule with the end of the biotin molecule of the DNA solid phase-ized film 15 formed in the specific part 101.
By passing through the process of the above (1) - (10), the DNA microarray substrate 100 with which avidin was fixed only to the specific part 101 of the front face of the slide glass substrate 11 by the monolayer is obtained. Spacing of 200 microns or less and part 101 specific comrades of the diameter of the specific part 101 is 400 microns or less.
[0008] The process which an avidin molecule combines with a monolayer is shown in the end of the biotin molecule of each DNA solid phase-ized film 15 at drawing 2 . Since all of a specific area and the specific configuration of a part 101 which are formed in the front face of each substrate are homogeneity, its number of the biotin molecules 23 fixed to each specific part 101 is also almost uniform. Therefore, the number of the avidin molecules combined with each specific part 101 becomes equal. That is, even if some dispersion is in the number of the biotins fixed to each specific part 101, the number of the avidin molecules with which the direction of an avidin molecule is fixed to each specific part 101 since it is larger than a biotin molecule for whether your being Haruka becomes fixed. Spacing of the configuration of the specific part 101, a dimension, and part 101 specific comrades can be changed into arbitration by changing the photo mask used at an exposure process. Therefore, the number of the avidin molecules fixed to the specific part 101 is also controllable to arbitration by changing a photo mask.
[0009] Next, the DNA microarray concerning this invention is explained. The DNA microarray concerning this invention is manufactured by each specific part 101 of the surface-preparation substrate 100 manufactured by the approach of drawing 1 by carrying out the spot of the solution containing the probe DNA from which a base sequence differs respectively. An example of the manufacture approach of the DNA microarray which starts this invention at drawing 3 is shown. 110 are AREIYA for carrying out the spot of the solution 111 containing probe DNA 21 on the DNA microarray substrate 100 among drawing. DNA (biotin-ized DNA) which carried out the indicator of the biotin beforehand is used for probe DNA 21. AREIYA 110 is supplied to the part 101 of specification [the solution 111 of a constant rate] by dashing the tip of AREIYA 110 which it had come to be able to carry out the constant-rate maintenance of the solution 111 by capillarity, and held the solution 111 against the specific part 101 on the surface treatment substrate 100. Consequently, the probe DNA 21 in a solution 111 combines with each one avidin molecule 22 of every currently fixed to the specific part 101 on the DNA microarray substrate 100 (solid-phase-izing of biotin-ized DNA). Since the number of the avidin molecules 22 currently fixed to each specific part 101 is the same, the number of the probe DNA 21 combined with each specific part 101 is also the same (refer to drawing 4). The number of the avidin molecules fixed to each specific part 101 can be changed into arbitration by changing the photo

mask used at an exposure process, and changing the specific configuration and specific dimension of a part 101, as mentioned above. Therefore, it is controllable by modification of the photo mask used at an exposure process to arbitration, and the number of the probe DNA 21 fixed to each specific part 101 can also always fix a fixed number of probe DNA 21 to each specific part 101, even if dispersion is in the probe DNA concentration of the solution 111 which carries out a spot to each specific part 101.

[0010] The example of measurement of the relation between the amount of DNA in the solution which carries out a spot to drawing 5 to the specific part on a surface treatment substrate (concentration), and the amount of DNA fixed by the specific part is shown. This measurement result shows that the amount of probe DNA fixed by the specific part becomes fixed, if the spot of the 3x109 to 5x109 probe DNA is carried out. All the DNA detection spot sections on a DNA microarray are the same configuration and the same area, and since same number [every] probe DNA 21 is moreover being fixed to all the detection spot sections, according to this DNA microarray, quantitive analysis is attained. Since the DNA adsorption site on a DNA microarray is restricted to the specific part 101, i.e., the DNA detection spot section, DNA can prevent that an un-unique target causes adsorption around the DNA detection spot section. Therefore, a S/N ratio can be raised by reduction of the noise at the time of fluorescence detection (unnecessary light). Furthermore, a S/N ratio can be raised more by making it in agreement with the configuration of the pixel of the solid state image sensors (a CCD sensor, CMOS sensor, etc.) which use the configuration of the DNA detection spot section, i.e., the configuration of said specific part, for an image pick-up.

[0011] In addition, this invention is not limited to the gestalt of the above operation. For example, the manufacture approach of the surface treatment substrate concerning this invention is not limited to the gestalt of the above-mentioned operation. That is, by the manufacture approach shown in drawing 1 , the laminating of the photoresist 13 of the aluminum film 12 and a positive type is first carried out to the front face of a substrate 11 one by one. Next, it lets the photo mask which has arranged the specific part 101 regularly pass, and only the specific part of a photoresist is exposed, by dipping in a developer, a photoresist dissolves only the specific part 101 and the aluminum film 12 exposes only the specific part 101. Then, by etching the aluminum film 12 with an acid etching solution, the aluminum film 12 of the specific part 101 is dissolved, and the glass front face of the specific part 101 of a substrate 11 is exposed. It can leave the DNA solid phase-ized film 15 only to the specific part 101 of the front face of a substrate 11 by applying the DNA solid phase-ized film 15 on it, and dissolving the aluminum film 12. As another approach, the DNA solid phase-ized film 15 may be formed in the whole front face of a substrate 11 from the beginning, and only the specific part 101 may adopt the approach of exposing the DNA solid phase-ized film 15. In this case, after carrying out laminating formation of the DNA solid phase-ized film 15 and the aluminum film 12 one by one on the whole front face of a substrate 11, only the specific part 101 can expose the DNA solid phase-ized film 15 by carrying out laminating formation of the photoresist 13 of a positive type on the aluminum film 12, exposing only to the specific part 101 through a photo mask, and performing development and etching like the above. Moreover, a photoresist does not need to be a positive type and it is undoubted that the photoresist of a negative mold is also usable.

[0012] Moreover, although the gestalt of the above-mentioned implementation explained the DNA microarray, i.e., the biomolecule microarray which fixed DNA as probe biomolecule, the thing using RNA, PNA, protein, etc. as probe biomolecule is also contained in the biomolecule microarray of this invention. Moreover, the substrate used for a surface treatment substrate may not be restricted to a slide glass substrate, and a transparence glass substrate, a silicon substrate, a plastic plate, a golden substrate, a silver substrate, etc. are sufficient as it. Moreover, what is necessary is just to use the matter considered to be the most suitable for quantification of the number of probe biomolecules to fix in consideration of affinity with probe biomolecule to fix not only to what fixed avidin (exposure) but to said specific part, even if attached to a solid phase-ized agent.

[Translation done.]

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DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] It is the production process Fig. showing an example of the manufacture approach of the surface treatment substrate concerning this invention.
[Drawing 2] It is an explanatory view about the process which an avidin molecule combines with the end of the biotin molecule of each DNA solid phase-ized film.
[Drawing 3] It is the explanatory view showing an example of the manufacture approach of the DNA microarray concerning this invention.
[Drawing 4] It is an explanatory view about the process which probe DNA (biotin-ized DNA) combines with the avidin molecule currently fixed to the specific part.
[Drawing 5] It is drawing showing the measurement result of the relation between the amount of DNA in the solution which carries out a spot to the specific part on a surface treatment substrate (concentration), and the amount of DNA fixed by the specific part.
[Description of Notations]
11: Slide glass substrate
12: Aluminum film
13: Photoresist
14: Photo mask
15: Solid phase-ized film
21: Probe DNA
22: Avidin molecule
23: Biotin molecule
100: DNA microarray surface treatment substrate
101: A specific part
110: AREIYA
111: Solution

[Translation done.]

* NOTICES *

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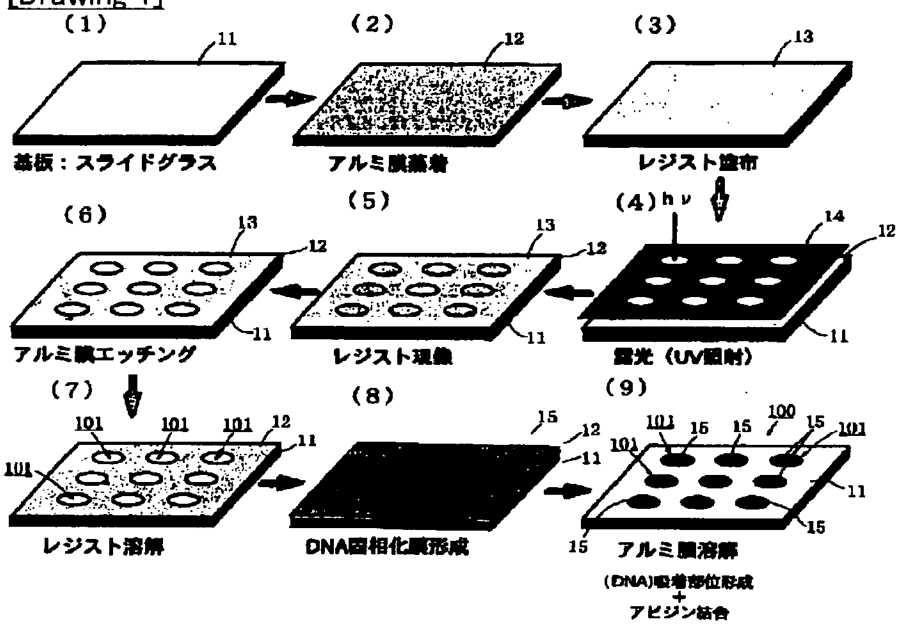
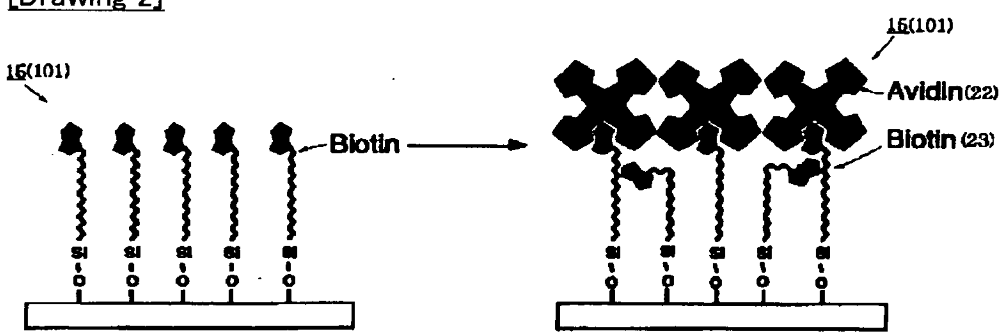
1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DRAWINGS

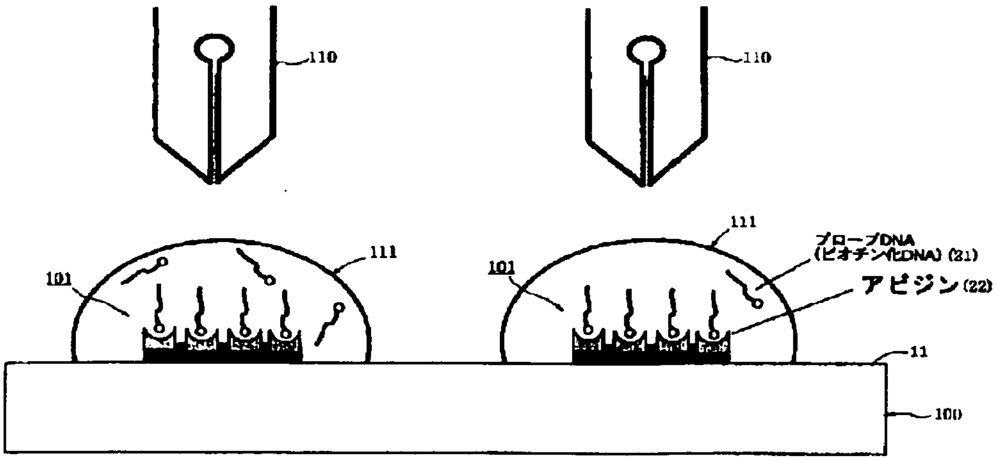
[Drawing 1]



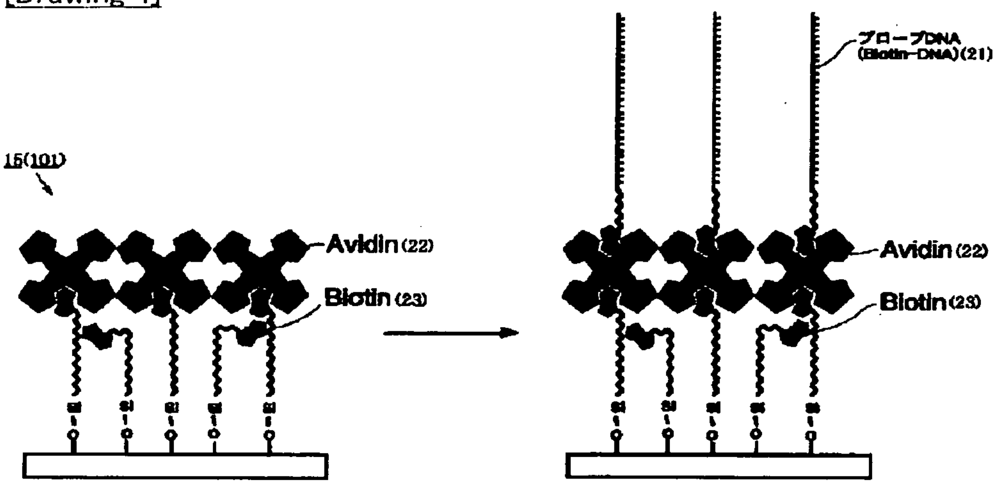
[Drawing 2]



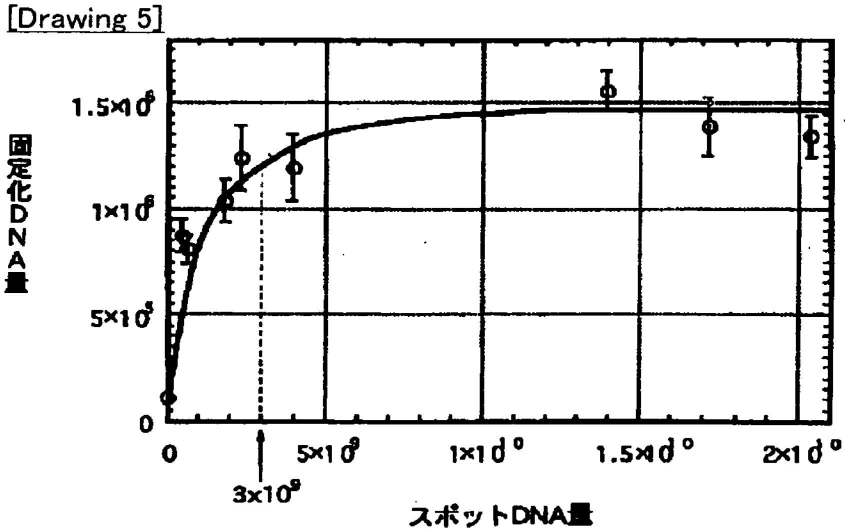
[Drawing 3]



[Drawing 4]



[Drawing 5]



[Translation done.]

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(71) Applicant *(for all designated States except US)*: CIBA-GEIGY AG [CH/CH]; Klybeckstrasse 141, CH-4002 Basle (CH).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: FELDER, Eduard [CH/CH]; Schützenmattstrasse 52, CH-4051 Basle (CH). RINK, Hans [CH/CH]; Rebenstrasse 10, CH-4125 Riehen (CH). MATTHEWS, Ian, Timothy, William [GB/GB]; 7 Wimblehurst Road, Horsham, West Sussex RH12 4EA (GB).

(74) Common Representative: CIBA-GEIGY AG; Patentabteilung, Klybeckstrasse 141, CH-4002 Basle (CH).

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(54) Title: PROCESS FOR THE PRODUCTION OF COMBINATORIAL COMPOUND LIBRARIES

(57) Abstract

The present invention relates to a process for the preparation of a plurality of different units consisting of a solid or semisolid carrier (bead), a synthetic oligomer (ligand) and an identification structure (tag) by means of which the monomers of the ligands are coded, and the use of said library for searching for novel classes of compounds and individual compounds. The invention further relates to compounds found with the novel process and the use thereof as thrombin inhibitors.

Process for the production of combinatorial compound libraries

The present invention relates to a process for the preparation of a plurality of different units consisting of a solid or semisolid carrier (bead), a synthetic oligomer (ligand) and an identification structure (tag) encoding the monomers of the ligands, and to the use of said library for searching for novel classes of compounds and individual compounds. The invention further relates to compounds detected with the novel process and to the use thereof as thrombin inhibitors.

Recently there has been an ever-increasing demand for chemical compounds that bind selectively only to a specific receptor. These compounds can then be used, inter alia, as inhibitors, agonists, antagonists, or for marking. In this connection, the possibility has been developed of simultaneously synthesising a multiplicity (library) of different compound and assaying them for their ability to bind to a specific receptor. These libraries (combinatorial compound libraries =CCL) usually consist of natural amino acids or of nucleotides and, ever more frequently, also of modified amino acids, modified nucleotides and other chemical compounds which are used as monomer building blocks.

The individual different ligands of these libraries are normally synthesised in parallel, either as mixtures or as physically separated individuals. These parallel syntheses make it possible to produce libraries containing a very large number of different ligands over a shorter period of time.

The literature describes two intrinsically different main strategies for the chemical synthesis of libraries consisting of a plurality of test candidates.

1. The ligands are obtained after their synthesis together in solution (Houghten *et al.* Nature (1991) **354**, 84-86).
2. The ligands are synthesised on a solid carrier, the synthesis being controlled such that only one species of ligand is bound to the carrier (one bead one sequence; Lam *et al.* Nature (1991), **354**, 82-84; Furka *et al.*, 14^{th} Intern. Congr. Biochem. FR013, 1988).

In the first strategy the problem consists in clearly identifying the desired ligand, as all ligands are present simultaneously in the solution. To identify ligands with a number of variable positions, it is necessary to synthesise a pool for each possible monomer per variable position, so that for a ligand having 4 variable positions, each of which may be

occupied by one of 20 amino acids, 4×20 = 80 pools have to be synthesised and assayed.

The second strategy produces all desired possibilities in a single pool and permits simple separation of the solid carriers containing the desired ligands and hence the identification of the ligands. The limitation here, however, resides in the type of ligands used, as their composition must subsequently be identified. Accordingly, only ligands are suitable that can be clearly identified by sequencing (peptides from naturally occurring amino acids, DNA and RNA). A further drawback is that the attachment of the ligands to solid carriers can lead to false-positive results, i.e. many assays cannot be carried out with ligands that are attached to solid carriers.

In contrast to these known possibilities, the present invention provides a library in which to each solid carrier only one type of ligand (one bead, one sequence) and one identification structure coding for this ligand (tag) is bound. The clear assignment of one ligand to one tag makes it possible to identify in a binding assay also those ligands which, in the amounts in which they are present on a carrier, cannot be unequivocally identified by known methods and/or by automated procedures. A further surprising advantage is that the ligand can be separated from the carrier for a further assay without loss of identifiability in its entirety or in portions, so that the results of the assay are in no way influenced by the solid carrier. With the identified ligands that are present in homogeneous solution it is thus possible to carry out assays which are not possible with the ligands bound to the carrier, for example further enzymatic assays or purity assays by IR, NMR or MS.

Accordingly, the invention relates to a library comprising a plurality of different units, each consisting of a solid or semisolid carrier (beads), a synthetic oligomer (ligand) and an identification structure (tag) by means of which the monomers of the ligand can be indentified, wherein:

a) each carrier unit carries only one type of ligand,
b) ligand and tag are attached to the same carrier at different positions,
c) the ligand, irrespective of the tag and without altering its information content, can be separated from the carrier,
d) the tag is a sequenceable polypeptide, and
e) to synthesise the tag or ligand a protective group is used that can be removed under mild acid conditions.

It is thus possible by means of the inventive process to carry out two different assays:

a) a first assay to preselect the ligand bound to the acceptor, said ligand being bound to the solid or semisolid carrier,

b) a second assay, after separation of the ligand from the carrier and the information structure, for the further characterisation of the ligand. The second assay can likewise investigate the attachment of the ligand to the acceptor or can be a completely different assay which cannot be carried out with the attached ligands.

Protective groups which can be removed under mild acidic conditions can be, for example, completely removed at concentrations of up to 10 %, preferably of up to 5 %, of acetic acid. These preferred protective groups will be at least 10x more labile than the dimethyl-dimethoxybenzyloxycarbonyl protective group (Ddz). Particularly preferred groups are those of the trityl type, such as unsubstituted or alkoxy-substituted trityl.

The term ligand embraces all compounds specifically comprising a plurality of monomers. Typically the monomers contain at least two reactive groups. Illustrative examples of such reactive groups are amino, azido, isocyanido, isocyanato, hydrazino, carbonyl, carboxyl, acylhalogeno, hydroxyl, sulfhydryl, sulfonyl chloride, phosphate groups, and halogeno groups. To facilitate the synthesis, the reactivity of the groups can be modified by protective or activating groups. Typical examples of monomers containing two or more reactive groups are natural and non-natural amino acids, ω-aminocarboxylic acids, saccharides, nucleotides and nucleotide analogs. When using monomers containing more than two reactive groups it is also possible to insert optional branches and cyclisations of the ligand. Monomers containing only one reactive group can be used as end groups.

The inventive process characterised in detail hereinbelow also makes it possible to produce libraries of ligands in which the individual building blocks are linked not only in succession, but also side by side, in a specific combination to a basic compound. This can be done by attaching a basic building block that contains a number of reactive groups direct or through a linker to the carrier and then attaching to this basic building block individual monomers that in turn contain one or more than one reactive group. Basic building blocks in the context of this invention are typically steroid nuclei, the penicillin or penem nucleus, soraphens, benzodiazipines, saccharides or desferoxiamine. Different reactive groups can then be fixed to these nuclei by standard chemical methods.

Preferred ligands contain monomers that cannot be identified clearly by sequencing

without further experimental effort, e.g. by known automated procedures.

Monomers that are not clearly identifiable by sequencing are all chemical compounds except the naturally occurring 20 amino acids and the nucleotides naturally occurring in DNA and RNA. Typical examples are modified amino acids or nucleotides, ω-aminocarboxylic acids, D-amino acids, saccharides, amino acids having saccharide side-chains, and end groups carrying only one reactive group such as acetyl or benzyl.

The ligand is usually attached to the solid or semisolid carrier through a linker. Linkers for the ligand are typically chemical compounds containing at least two reactive groups. Preferred linkers are those that tolerate mild basic as well as mild acidic conditions, so that ligands and tags can not only be synthesised but also assayed on the carrier. The preferred linkers are therefore cleavable only by means of a specific reaction, for example methionine, which is cleavable with cyanogen bromide, or linkers which are cleavable only under strongly basic or strongly acidic conditions, photolytically, or under reductive or oxidative conditions. Particularly preferred linkers are those that form cleavable bonds under basic, but stable bonds under acidic, conditions, so that the ligand can be removed selectively from the carrier. Illustrative examples of suitable linkers are p-hydroxymethylbenzoic acid, 4-hydroxymethylphenylacetic acid, benzhydrylamino, allyl, hydroxycrotonylaminomethyl, 3-nitro-4-hydroxymethylbenzoic acid, p-nitrobenzhydrylamine, 4-[4,4'-bis(methylsulfinyl)-2-oxybenzhydrylamino]butyric acid and disulfide linkers that in turn can be linked through a group called in solid-phase synthesis a "handle". These linkers suitable for use in the inventive process may contain, in addition to the described cleavable building blocks, further building blocks that influence or do not influence the cleavage reaction, which are suitable for separating the variable part of the ligand spatially from the cleavable group.

Short peptides which have a specific site for a protease such as trypsin, yscα, yscF or the V8-protease may also be used as linkers.

Particularly preferred linkers are also those whose cleavage can be controlled, so that under suitable conditions only a specific part of the linker is cleaved, thereby affording the possibility of separating the ligands from the solid carrier in several portions, e.g. for further assays. Those linkers are very particularly preferred that can be cleaved by a volatile or gaseous agent such as ammonia, as these linkers - before the assay of the ligands - can be removed completely or almost free of residue.

To be able to cleave the different ligands in exactly defined and - despite the heterogeneity of the ligands - equal portions, it is possible for the linker between the carrier and the ligand to consist of a cleavable part, as indicated above, as well as a part which is the same for all ligands. Suitable building blocks of the constant part may be in general chemical compounds which permit attachment not only to the cleavable part of the linker, but also to another chemical building block or to the ligand. Illustrative examples are bifunctional chemical compounds such as amino acids. nucleic acids or invariable parts of the ligand.

The information structure (tag) is a sequenceable polypeptide whose coding units conveniently consist of the naturally occurring amino acids (Ala, Arg, Asn, Asp, Cys, Gln, Glu, Gly, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr and Val). It is preferred to use amino acids which carry no reactive side-groups and/or are readily identifiable during sequencing, typically Ala, Asn, Asp, Gly, Ile, Leu, Phe, Pro, Trp and Val. Particularly preferred amino acids are Ala, Asn, Asp, Gly, Leu, Phe and Pro. The tag can in turn be bound to the carrier through one of the above described linkers which is cleavable under specific conditions. It is important in this connection that the separation of the linker and the tag is possible independently of the other and that the separation of the ligand from the carrier does not modify the information of the tag.

If all 20 naturally amino acids are used, then it is possible to code 20 different monomers per variable site. If it is desired to encode more than 20 different monomers per variable site, then more than one amino acid is used for each coding unit. By using dipeptides it is thus possible to code up to 20^2 different monomers with 20 different amino acids.

To avoid misinterpretations when sequencing the tag, it is possible to use only the preferred amino acids listed above. These amino acids can then be used as di- or tripeptides to increase the number of codable monomers, i.e. each monomer of the ligand is coded by more than one amino acid in the tag.

In order to ensure that the tag influences the test result as little as possible and that the amount of ligand is as high as possible, the ratio of ligand to tag on a solid or semisolid carrier is desirably greater than 1, preferably from 2 to 100 and, most preferably, from 4 to 10.

For practical considerations, the tag can be provided with a starting and/or end sequence

that does not code for a monomer of the ligand, so that, when sequencing the tag, it is possible to identify exactly the beginning and end of the part coding for the ligand. Illustrative examples of such starting and end sequences are amino acids that do not code for a monomer of the ligand as well as di- or tripeptides of identical amino acids such as Val-Val or Leu-Leu-Leu.

The term "solid or semisolid carrier" will be understood as meaning macroscopic particles that are insoluble in the reaction media and to which both the ligand and the tag can be bound in sufficient amount. The amount of tag that can be bound to the carrier must be sufficiently large to produce a clearly identifiable signal (e.g. > 1 pmol) when sequencing the tag. If the tag contains radioactively marked monomers, then also less than 1 pmol of tag per carrier can be identified on account of the lower detection limit for radioactive compounds.

The binding of ligand and tag is effected by means of reactive groups at the surface of the carrier, e.g. amino, carboxyl, hydroxyl or halogen groups. These reactive groups are usually already constituents of the carrier, but they can also be applied or modified subsequently. The resins customarily employed in solid-phase synthesis can be used, for example those used in Merrifield peptide synthesis. They consist largely of a polystyrene molecule that is crosslinked by copoylmerisation with divinyl benzene. The molecules are additionally derivatised to attach the reactants in the solid-phase synthesis.

The invention also relates to a process for the preparation of the above described library, which comprises synthesising ligand and tag by an orthogonal and alternating synthesis.

The synthesis of the libraries of this invention comprises the steps

a) attaching the first unit of the tag (preferably in less than equivalent amount, i.e. less than 50 mol % based on the reactive groups on the carrier) and the first monomer of the ligand or the building block thereof or a linker for each to the solid or semisolid carrier;
b) optionally attaching further non-variable monomers to the ligand or further none-variable coding units to the tag;
c) dividing the solid or semisolid carrier into portions for the variable monomers of the ligand;
d) carrying out in each portion separately, in codable sequence, further modifications at the ligand or attaching another of the variable monomers possible at this site of the ligand as well as the unit of the tag coding for this step;

ligand as well as the unit of the tag coding for this step;

e) mixing the portions;

f) repeating steps b) to e) until the variable part of the ligand is completely synthesised; and

g) optionally attaching one or more than one further invariable monomer to the ligand or further non-coding units to the tag (q.v. reaction schemes 1 and 2);

using for the synthesis of tag or ligand a protective group which can be removed under mild acidic conditions.

The type of the bond of the first unit of the tag and of the first monomer of the ligand and the linker for the first monomer depends on the type of chosen reactive group and is effected by the standard methods for such groups.

The further synthesis is carried out by standard known methods of solid-phase synthesis (Fields *et al.*, Intern. J. Pept. Prot. Res. (1990), 35, 161-214). The transient protective groups (protective end groups which can be eliminated before each synthesis step) used for the synthesis of ligand and tag are orthogonal protective groups, i.e. the syntheses of ligand and tag can be carried out independently of each other. For example, two groups of protective groups can be used, the first group being removable under mild acidic and the other group under basic conditions, by the action of light, under oxidative or reductive conditions. It is particularly preferred to use a combination of protective groups that are removable under mild acidic conditions and protective groups that are removable under basic conditions.

To ensure compatibility with a large number of protective groups which shall remain intact during the ligand synthesis, the acid-removable transient protective groups are those having especially pronounced acid lability. Suitable acid-labile protective groups are at least 10x more labile than the dimethyldimethoxybenzyloxycarbonyl protective group (Ddz).

To achieve full orthogonality, base-removable fluorenylmethoxycarbonyl (Fmoc) is preferably used for the synthesis of the one structure, and acid-removable trityl (Trt) or substituted trityl protective groups such as alkoxy-substituted trityl for the synthesis of the other structure.

Illustrative examples of the combination of protective group types are:

Ligand	Tag	Side-chains of the monomers
Fmoc type	Trt type	acid-labile or allyl type
Trt type	Fmoc type	acid-labile or allyl type
allyloxycarbonyl	Trt type	acid-labile
Trt type	allyloxycarbonyl	acid-labile
Trt type	Boc or NPS type	allyl type
Boc or NPS type	Trt type	allyl type
allyloxycarbonyl	Trt type	β-elimination type
Trt type	allyloxycarbonyl	β-elimination type

Groups of the β-elimination type are typically protective groups of the fluorenylmethyl type; NPS denotes groups of the nitrophenylsulfenyl type. A general description of the eligible groups will be found in Fields *et al.* (Intern. J. Pept. Prot. Res. (1990), **35**, 161-214).

Also suitable are combinations of one of the above groups of protective groups for ligands or tags with protective groups which can be removed by photolysis, e.g. nitrobenzyl or nitroveratryloxycarbonyl groups.

The reaction steps required e.g. for the synthesis of amides are widely known in the art and usually depend on the type of activation of the carboxylic acid group participating in the reaction. The reactions normally run in the presence of a condensing agent or, when activating the carboxylic acids in the form of anhydrides, of an agent that binds the carboxylic acid formed. In some cases it is also possible to add chaotropic reagents such as LiF in NB-methylpyrrolidone. The reactions are carried out in the temperature range from -30°C to +150°C, preferably from +10°C to +70°C and, most preferably, from +20°C to +50°C, and, if appropriate, also in an inert gas atmosphere.

Illustrative examples of useful condensing agents are carbodiimides such as N,N'-diethyl-, N,N'-diisopropyl-, N,N'-dicyclohexyl- or N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide; carbonyl compounds such as carbonyl diimidazole; 1,2-oxazolium compounds such as 2-ethyl-5-phenyl-1,2-oxazolium-3'-sulfonate and 2-tert-butyl-5-methylisoxazolium perchlorate; acylamino compounds such as 2-ethoxy-1-ethoxycarbonyl-1,2-dihydroquinoline; and uronium compounds such as 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetra-

methyluronium tetrafluoroborate (TBTU); or phosphonium compounds such as benzotriazol-1-yloxy-tris(dimethylamino)phosphonium hexafluorophosphate (BOP) or benzotriazol-1-yl-oxy-pyrrolidinophosphonium hexafluorophosphate (PyBOP).

Useful acid acceptors are typically alkali metals, carbonates or bicarbonates, such as sodium or potassium carbonate or bicarbonate (usually together with a sulfate), or organic bases such as sterically hindered tri-lower alkylamines such as N,-diisopropyl-N-ethylamine.

Reactive side-chains of the monomers of the ligand and tag which shall not participate in the reactions may be protected by a third group of protective groups. Useful protective groups and processes for their introduction and removal are described, inter alia, in "Protective Groups in Organic Chemistry", Plenum Press, London, New York 1973; "Methoden der organischen Chemie", Houben-Weyl, 4th edition, Vol. 15/1, Georg-Thieme-Verlag, Stuttgart 1974; Th. W. Greene, "Protective Groups in Organic Synthesis", John Wiley & Sons, New York 1981; Atherton *et al.*, "Solid phase peptide synthesis-A practical approach" IRL Press Oxford University, 1984; Jones, "The chemical synthesis of peptides", Oxford Science Publications, Clavendon Press Oxford, 1991; und Bodanszky, "Peptide Chemistry", Springer Verlag Berlin, 1988.

Typical examples of hydroxy protective groups are acyl radicals such as the tert-butoxycarbonyl radical, etherifying groups such as the tert-butyl group, and silyl or tin radicals such as the tri-n-butyltin radical or tert-butyldimethylsilyl.

Carboxyl groups may be protected by ester formation with groups of the tert-butyl type, benzyl, trimethylsilylethyl or 2-triphenylsilyl groups.

Amino groups may conveniently be protected by removable acylamino, arylmethylamino, esterified mercaptoamino, 2-acylalk-1-enylamino, silylamino, tin amino or azido groups, including tert-butoxycarbonyl, allyloxycarbonyl, benzyloxycarbonyl, 4-nitrobenzyloxycarbonyl, diphenylmethoxycarbonyl, nitrophenylsulfenyl, 2,2,2-trichloroethoxycarbonyl, pentamethylchromanosulfonyl (PMC) or methoxytrimethylbenzylsulfonyl (Mtr) protective groups.

Thiols may be protected by acetamidomethyl groups.

These protective groups are usually removed after the complete synthesis of ligand and tag jointly by conventional methods of peptide chemistry, conveniently by treatment with 95 % trifluoroacetic acid. In some cases strong nucleophiles, such as 1,2-ethanedithiol, may additionally be added to capture the generated protective groups.

Further methods of removing these protective groups are known and comprise, inter alia, β-elimination, solvolysis, hydrolysis, alcoholysis, acidolysis, treatment with a base or reduction.

The number of portions for the reactions described in d) preferably corresponds in each case to the number of possibilities of the next monomer of the ligand to be attached. As stated above, one unit of the tag can consist of one or more than one amino acid. If a unit consists of several amino acids, each of these can be attached in succession to the growing tag or synthesised separately as unit and then attached in a reaction to the tag. Sequencing of the tag can be made easier by additionally attaching to the beginning and end thereof one or more than amino acid which indicates the start or end of the part carrying the information (start sequence/end sequence).

To identify the ligand which binds to the desired acceptor, the library prepared above is treated with the acceptor to be investigated, and those carriers to which the acceptor remains attached are washed and isolated.

Possible acceptors within the scope of this invention are macromolecular units which have affinity for binding to one or more than one ligand. Illustrative examples are receptors including serotonin receptor, GABA receptor and benzodiazepine receptor; transport proteins such as Na and K channels, antibodies and enzymes such as proteases, thrombin, renin, ACE, aromatase and reverse transcriptase; or fragments thereof which have the same binding properties for the ligands. The ligands binding to these acceptors act as antagonists, inhibitors, agonists, or as markers for the acceptors.

Unless not already naturally present, the acceptors are provided with an identifiable group such as a fluorescing, chemoluminescing or radioactive group, avidine, biotine, reporter enzymes, immunologically detectable groups (ELISA) or the like. Methods of marking with the markers referred to above and of affinity chromatography are generally known in the art.

The isolation of the carriers to which an acceptor is bound is effected manually by sorting the carriers, e.g. under UV or blue light in the case of fluorescing acceptors, or with the aid of automated apparatus typically used for sorting cells.

The tag of each carrier isolated in this manner can be sequenced with the conventional methods of peptide analysis, e.g. Edman degradation, and the ligand attached to said carrier clearly identified. Sequencing can be carried out before or after separation of the ligand, e.g. by an automated sequencing apparatus, at the tag attached to the carrier. Sequencing after separation of the ligand is preferred, as in this case liberated monomers of the ligand do not interfere with the sequencing. Another possibility consists in derivatising the ligands terminally in suitable manner (e.g. by acetylation), so that they are not degraded by the analysis of the tag.

A further possibility consists in separating the tag from the carrier and then sequencing the tag.

To rule out false-positive results of the binding test, the ligands can be separated wholly or partially from the individual carriers and, in homogeneous solution, subjected to a further assay, e.g. a second round of assays for their attachment to the acceptor. The separation of the ligand is effected e.g. by cleavage at the linker between the carrier and the ligand by a reaction specific to said linker. If the linker is p-hydroxybenzoic acid, this cleavage can be effected by treatment of the individual carriers with gaseous ammonia, a saturated solution of ammonia/THF or liquid ammonia (e.g. in a microtitration plate). The separated ligand can then be rinsed from the carrier and once more tested for its bonding to the acceptor, or a further assay is carried out which cannot be performed with the ligands bound to the carrier, typically binding studies, tests in which light absorption is measured, or MS, IR or NMR investigations.

A suitable choice of linker also makes it possible, for a first assay, to separate a first portion of the ligands from the isolated carriers by treatment with gasesous ammonia, and a second portion of the ligands for a further assay by treatment with a solution of ammonia/THF or liquid ammonia. A suitable linker in this connection is typically 4-hydroxymethylbenzoic acid.

A typical example is the synthesis of a library of potential thrombin inhibitors which contain non-natural amino acids and other building blocks and are thus more

protease-resistant.

The library consists e.g. of pentamers having 2 constants and 3 variable positions and having the composition:

X - Y - Z - Pro - GABA

X= 2-cyanobenzenesulfonyl, D-Phe, N-benzylglycine, β-Ala or acetyl
Y= L-Pro, D-Pro, β-Ala or L-Asp
Z= L-Arg, D-Arg, β-Ala, L-Asp or sarcosyl

This composition affords $5 \times 4 \times 5 = 100$ possibilities for the composition of the ligands.

The individual monomers are coded by dipeptides in the identification structure.

After screening this library, it is found that the pentamers D-Phe-D-Pro-Arg-Pro-GABA and D-Phe-Pro-D-Arg-Pro-GABA are all effective thrombin inhibitors.

Accordingly, a further object of the invention is a compound of formula D-Phe-D-Pro-Arg-Pro-GABA, D-Phe-Pro-D-Arg-Pro-GABA, or pharmaceutically acceptable salts thereof, for inhibiting thrombin; and pharmaceutical compositions that contain said thrombin inhibitors, singly or in combination with further optional excipients.

The invention further relates to each compound detected by the novel process for use in a method of therapeutic and prophylactic treatment of the human or animal body. The preferred field of use is that of therapeutic and prophylactic treatment in connection with the thrombin function such as embolisms and thromboses.

The pharmaceutical compositions of this invention are those for enteral, e.g. oral, and also rectal and parenteral administration, for example subcutaneous, intravenous or intraperitoneal administration, to warm-blooded animals, and they contain the pharmacologically active compound alone or together with a pharmaceutically acceptable carrier. The daily dose will depend on the age and individual condition of the patient as well as on the mode of administration.

The novel pharmaceutical compositions contain from about 10 to 80 %, preferably from about 20 to 60 %, of the active compound. Pharmaceutical compositions for enteral or parenteral administration are typically those in dosage unit forms such as dragées, tablets, capsules or suppositories, and also ampoules. These dosage forms are prepared in a manner known per se, typically by conventional mixing, granulating, confectioning, dissolving or lyophilising methods.

Pharmaceutical compositions for oral administration are preferred. Suitable carriers are especially fillers such as sugars, conveniently lactose, saccharose, mannitol or sorbitol, cellulose preparations and/or calcium phosphates, typically tricalcium phosphate or calcium hydrogen phosphate, and also binders such as starch pastes, conveniently using maize, corn, rice or potato starch, gelatin, tragacanth, methyl cellulose and/or polyvinyl pyrrolidone, and/or, if desired, disintegrators such as the above-mentioned starches, also carboxymethyl starch, crosslinked polyvinylpyrrolidone, agar, alginic acid or a salt thereof such as sodium alginate. Excipients are in particular glidants, flow control agents and lubricants, conveniently silica, talcum, stearic acid or salts thereof, typically magnesium stearate or calcium stearate, and/or polyethylene glycol. Dragée cores can be provided with suitable non-enteric or enteric coatings, typically using concentrated sugar solutions which may contain gum arabic, talcum, polyvinylpyrrolidone, polyethylene glycol and/or titanium dioxide, shellac solutions in suitable organic solvents or mixtures of solvents or, for the preparation of enteric coatings, solutions of suitable cellulose preparations such as acetyl cellulose phthalate or hydroxypropylmethyl cellulose phthalate. Dyes or pigments can be added to the tablets or dragée coatings, conveniently to identify or indicate different doses of active compound.

Further pharmaceutical compositions for oral administration are dry-filled capsules made of gelatin and also soft-sealed capsules consisting of gelatin and a plasticiser such as glycerol or sorbitol. The dry-filled capsules can contain the active ingredient in the form of granules, conveniently in admixture with fillers such as lactose, binders such as starches, and/or glidants such as talcum or magnesium stearate, and with or without stabilisers. In soft capsules, the active ingredient is preferably dissolved or suspended in a suitable liquid, typically a fatty oil, paraffin oil or a liquid polyethylene glycol, to which a stabiliser can also be added.

Suitable pharmaceutical compositions for rectal administration are typically suppositories, which consist of a combination of the active compound with a suppository base. Examples

of suitable suppository bases are natural or synthetic triglycerides, paraffin hydrocarbons, polyethylene glycols and higher alkanols. It is also possible to use gelatin capsules for rectal administration that contain a combination of the active compound with a base substance. Suitable base substances are typically liquid triglycerides, polyethylene glycol or paraffin hydrocarbons.

Pharmaceutical compositions for parenteral administration contain aqueous solutions or suspensions of the active compound, conveniently oily injection suspensions using suitable lipophilic solvents or vehicles such as fatty oils, typically sesame oil, or synthetic fatty acid esters such as ethyl oleate or triglycerides, or aqueous injection suspensions which may contain viscosity increasing substances, conveniently sodium carboxymethyl cellulose, sorbitol and/or dextran, and also with or without stabilisers.

The invention also relates to the use of the above mentioned thrombin inhibitors, preferably in the form of pharmaceutical compositions. The dosage of the active compound will depend on the species of the warm-blooded animal, on the age and individual condition of the patient, and also on the mode of administration. The contemplated daily dosage for parenteral administration to a patient of approximately 75 kg body weight will be from about 0.1 mg to 500 mg, preferably from about 1 mg to 50 mg.

The invention is illustrated in more detail by the following Examples.

The following abbreviations are used:

DCCI = dicyclohexylcarbodiimide
DCE = dichlorethane
DICD = diisopropylcarbodiimide
DIPEA = diisopropylethylamine
DMA = dimethylacetamide
DMF = dimethylformamide
DMAP = dimethylaminopyridine
DMSO = dimethylsulfoxide
Fmoc = fluorenylmethyloxycarbonyl
GABA = γ-aminobutyric acid
HOBT = hydroxybenzotriazole

NEt_3	= triethylamine
OSu	= O-succinimide
Pip	= piperidine
PMC	= pentamethylchromanesulfonyl
Trt	= trityl

Repetitive treatments are carried out in accordance by the batch process with the procedures of the solid-phase technique commonly used in peptide chemistry (Fields *et al.*, Intern. J. Pept. Prot. Res. (1990), 35, 161-214), e.g. by addition of reagent solutions to the resin and subsequent filtration (isolation of the filtrate under a weak vacuum).

The preparation of a library (q.v. reaction scheme 1), consisting of pentamers having 2 constant and 3 variable positions and having the composition

X - Y - Z - Pro - GABA

X = 2-cyanobenzenesulfonyl, D-Phe, N-benzylglycine, β-Ala, acetyl
Y = L-Pro, D-Pro, β-Ala, L-Asp
Z = L-Arg, D-Arg, β-Ala, L-Asp, sarcosyl

is described

The individual monomers described above are coded by the following dipeptides in the identification structure:

Building blocks	Dipeptide
2-cyanobenzenesulfonyl chloride	DG
D-Phe	GD
N-benzylglycine	GF
β-Ala	AA
Acetyl	AG
L-Pro	DA
D-Pro	GA
L-Arg	GG

D-Arg	NG
L-Asp	AN
sarcosyl	NA

Example 1: Preparation of the trityl-protected amino acids and dipeptides
10 mmol of each dipeptide or amino acid are suspended in 36 ml of chloroform/acetonitrile (4:1). Then 10 mmol of trimethylsilyl chloride are slowly added dropwise, and the reaction mixture is refluxed for 2 hours at c. 65°C. Then 20 mmol of triethylamine are added dropwise, and finally a solution of 10 mmol of trityl chloride in 10 ml of chloroform is added. After 60 minutes methanol is added in excess and, after a further 5 minutes, the reaction mixture is concentrated by evaporation and the residue is dried. The crude material is taken up in an ice-cooled 2 M solution of potassium hydrogensulfate (or citric acid) and the solution is extracted with ethyl acetate. After back-extraction with aqueous alkali (at c. 5°C), the aqueous phase is adjusted with potassium hydrogensulfate solution to pH 6-7. The Trt-dipeptide or the Trt-amino acid precipitates and is extracted with ethyl acetate. 10 mmol of triethylamine are added to the organic phase and the batch is concentrated by evaporation (triethylammonium salt of Trt-dipeptide or Trt-amino acid),

The triethylammonium salts of the Trt-dipeptides DG, GD, GF, AA, AG DA, GA, GG, NG, AN and NA as well as the Trt-amino acids of A, D, F, G and N are prepared in this manner.

Example 2: Partial derivatisation of the carrier resin with a less than equivalent amount of a constant unit of the tag
181 µmol of reactive alkylamino groups of a Polyhipe SU 500® resin (Novabiochem), derivatised with ethylenediamine, are each washed 5x for 40 seconds with 20 % Pip/DMA and afterwards 10x for 60 seconds with DMA at room temperature.

Loading: c. 430 µmol/g are equivalent to c. 2000 pmol/resin particles (bead)

For preactivation, 60 µmol of Trt-Gly-OH·NEt_3 of Example 1 are dissolved in 66 µmol (based on HOBT) of a 0.5 M solution of HOBT/DMA and to this solution are added 66 µmol (based on DICD) of a 2 M solution of DICD/DMA. After 40 minutes at room temperature, the mixture is diluted with 2 ml of DMA and the solution is pipetted onto the resin. Immediately afterwards, 66 µmol of DIPEA are added. The coupling reaction is continued for 60 minutes. The resin is rinsed 10x 45 seconds with DMA.

Example 2.1: Determination of the tag loading by measuring the free amino groups
The reporter group Fmoc is coupled to the resin and, after washing off excess amounts, split off again and measured.

Procedure:
275 µmol of Fmoc-OSu are dissolved in 2.1 ml of DMA and added to the resin. Immediately afterwards, 413 µmol (based on DIPEA) of a 1.5 M solution of DIPEA/DMA are pipetted onto the resin. After 80 minutes at room temperature, the resin is washed 10x 18 seconds with DMA. To remove the Fmoc group, the resin is treated 15x 40 seconds at room temperature with 20 % of Pip/DMA. The collected filtrates are diluted in defined manner and their extinction is measured at 299.8 nm. The total number of free amino groups is c. 154 µmol, i.e. 85 % of the total loading of the resin (percentage of ligand). The tag loading of the resin is thus c. 15%.

Example 3: Coupling the linker for the ligand to the resin
For preactivation, 769 µmol of 4-hydroxymethylbenzoic acid are dissolved in 845 µmol (based on HOBT) of a 0.5 M solution of HOBT/DMA and to the solution are added 845 µmol (based on DICD) of a 2 M solution of DICD/DMA. After 40 minutes at room temperature, the reaction mixture is diluted with 0.3 ml of DMA and pipetted onto the resin. Immediately afterwards, 845 µmol of DIPEA are added and the coupling reaction is continued for 60 minutes at room temperature. Afterwards rinsing is effected is follows:

10x 45 seconds with DMA
5x 60 seconds with isopropanol
6x 45 seconds with DMA

Example 4: Linking the first constant C-terminal component of the ligand with the linker

Batch: 154 µmol
The modified resin of Example 3 is pipetted to a solution of:

615 µmol	of Fmoc-GABA-OH
600 µl	of DMA and
3 ml	of dichloroethane

To this suspension is added a solution of:

646 µmol	of DCCI and
307 µl	of dichloroethane

in 2 portions over 5 minutes at room temperature and, finally, a solution of:

30.7 µmol of DMAP and
65 µl of dichloroethane

is added. After a total time of 20 minutes, 154 µmol of N-methylmorpholine are added. The reaction is continued for 4 hours and afterwards rinsing is effected as follows:

4x 45 seconds with DMA
3x 45 seconds with dichloroethane
4x 45 seconds with DMA.

Example 5: Chain lengthening of the constant part of the ligand

The Fmoc group is removed, as described in Example 2. Measurement of the extinction gives an amount of ligand of 122 µmol.

For preactivation, 367 µmol of Fmoc-Pro-OH are dissolved in 404 µmol (based on HOBT) of a 0.5 M solution of HOBT/DMA and to this solution are added 404 µmol (based on DICD) of a 2 M solution of DICD/DMA. After 40 minutes at room temperature, the reaction mixture is diluted with 1.3 ml of DMA and added to 122 µmol of resin of Example 4. Immediately afterwards, 404 µmol of DIPEA are added, and the coupling reaction is continued for 45 minutes at room temperature. A post-coupling with the same amount of Fmoc-Pro (as above) is carried out to increase the yield (another 35 minutes). The resin is rinsed and treated 1x 4 minutes with acetic anhydride/pyridine/DMA 1:1:8 (v:v:v) to block unreacted amino groups and subsequently rinsed as follows:

5x 45 seconds with DMA
5x 45 seconds with isopropanol.

The resin is dried and portioned into suitable pools for introduction of the variable positions. The introduction of the variable Z in this Example requires 5 portions for the separate reactions with 5 different building blocks ("split synthesis").

Example 6: Synthesis of the variable parts of the ligands and of the corresponding tag

Portioning into 5 pools each of 19 µmol is carried out (one pool for each variable possible at this position).

Example 6.1: Synthesis of the 1st variable position of the ligand

First the Fmoc group is removed at the ligand as described in Example 2. Then follow the separate coupling reactions for the 1st variable position (Z position):

Pool	Monomer	Amount	Coding
1	Fmoc-L-Arg(PMC)-OH	76 µmol	GG
2	Fmoc-D-Arg(PMC)-OH	76 µmol	NG
3	Fmoc-β-Ala-OH	76 µmol	AA
4	Fmoc-L-Asp(OtBu)-OH	76 µmol	AN
5	Fmoc-sarcosyl-OH	76 µmol	NA

Each of the above listed Fmoc building blocks is preactivated with 84 µmol of HOBT solution (0.5 M in DMA) and 84 µmol of DICD solution (2 M in DMA) for 40 minutes at room temperature and, after dilution with 230 µl of DMA, added to one resin portion. Immediately afterwards, 84 µmol of DIPEA are added. The coupling is carried out for 45 minutes at room temperature. A post-coupling is carried out for an additional 35 minutes with an identical mixture. Acetylation and rinsing are carried out as described in Example 5.

Example 6.2: Chain lengthening of the tag via dipeptides
In accordance with the code table, in each pool of Example 6.1, in accordance with the coupled monomer, the tag portion with the Trt peptide of Example 1 coding for the monomer is individually further synthesised (q.v. reaction scheme 2).

First the trityl groups of the tag irreversibly linked to the resin are removed by cleavage with 5 % formic acid (2x for 1 minute) in dichloroethane and rinsed:

5x 45 seconds with DMA
3x 45 seconds with 3 % triethylamine in DMA
3x 45 seconds with DMA.

Afterwards the dipeptides are linked to the respective tags in separate reactions.

1	Trt-GG	129 µmol
2	Trt-NG	129 µmol
3	Trt-AA	129 µmol
4	Trt-AN	129 µmol
5	Trt-NA	129 µmol

The above listed Trt-dipeptides are preactivated for c. 40 minutes in 142 µmol of HOBT and 142 µmol of DICD solution in DMA. Then the solutions are added to the respective resin portions and immediately treated with 142 µmol of DIPEA. The coupling reactions are continued for 60 minutes at room temperature and afterwards rinsed as follows:

2x 45 seconds with DMA
1x 5 minutes with acetic anhydride:pyridine:DMA = 1:1:8 (v:v:v)
5x 45 seconds with DMA.

<u>Example 6.3: Chain lengthening of the tag via individual amino acids (alternative to Example 6.2)</u>

First the trityl groups of the tag linked irreversibly to the resin are removed by cleavage with 5 % formic acid in dichloroethane, as described in Example 6.2.

For 5 portions of 4.3 µmol each of tag component

1:	Trt-G (I)	129 µmol
	Trt-G (II)	129 µmol
2:	Trt-G (I)	129 µmol
	Trt-N (II)	129 µmol
3:	Trt-A (I)	129 µmol
	Trt-A (II)	129 µmol
4:	Trt-N (I)	129 µmol
	Trt-A (II)	129 µmol
5:	Trt-A (I)	129 µmol
	Trt-N (II)	129 µmol

The Trt-amino acids listed above in (I) are preactivated for c. 40 minutes in 142 µmol of HOBT and 142 µmol of DICD solution in DMA. Then the solutions are added to the respective resin portions and immediately treated with 142 µmol of DIPEA. The coupling reactions are continued for 60 minutes at room temperature and afterwards rinsed as follows:

2x 45 seconds with DMA
1x 5 minutes with acetic anhydride:pyridine:DMA = 1:1:8 (v:v:v)
5x 45 seconds with DMA.

Afterwards the Trt groups are removed as shown above and each of the Trt-amino acids

listed above under (II) are coupled as indicated above.

Example 6.4: Synthesis of the 2nd variable position of the ligand

Position Y is synthesised in general accordance with the introduction of Z (with respect to the different codings) (q.v. reaction scheme 2). This is done by mixing all samples of Example 6.2 or 6.3 and dividing them into 4 portions of equal size (4 Y portions).

In these 4 separate portions, each of the monomers possible at this position is attached as described in Example 6.1

Pool	Monomer	Amount	Coding
1	Fmoc-L-Pro-OH	76 μmol	DA
2	Fmoc-D-Pro-OH	76 μmol	GA
3	Fmoc-β-Ala-OH	76 μmol	AA
4	Fmoc-L-Asp(OtBu)-OH	76 μmol	AN

As described in Example 6.2 or 6.3, the unit of the tag belonging to the monomer is then attached.

Example 6.5 Synthesis of the 3rd variable position of the ligand

Position X is synthesised in general accordance with the introduction of Y or Z (with respect to the different codings) (q.v. reaction scheme 2). This is done by mixing all samples of Example 6.4 and dividing them into 5 portions of equal size (5 X portions).

In these 5 separate portions, each of the monomers possible at this position is attached as described in Example 6.1

Pool	Monomer	Amount	Coding
1	2-cyanobenzenesulfonyl chloride	76 μmol	DG
2	Fmoc-D-Phe-OH	76 μmol	GD
3	Fmoc-N-benzylglycine-OH	76 μmol	GF
4	Fmoc-β-Ala-OH	76 μmol	AA
5	acetic acid anhydride	76 μmol	AG

The coupling with 2-cyanobenzenesulfonyl chloride is done via a reaction with a solution of the sulfonylchloride in pyridine/DMA at room temperature; the coupling of acetic acid anhydride is done as described in Example 5.

As described in Example 6.2 or 6.3, the unit of the tag belonging to the monomer is then attached.

Example 7: Deprotection of the ligand

An appropriate number of resin particles of the library obtained in protected form (but a multiple of the component number) is subjected to the deprotection reactions commonly employed in peptide chemistry to remove the Fmoc and tert-butyl protective groups. To remove the Fmoc protective groups, the resin of Example 6.5 is again treated repeatedly with 20 % Pip/DMA (q.v. Example 2) and then, to remove the t-butyl and Pmc protective groups, with 95 % trifluoroacetic acid (5 % water + 2% ethanedithiol) for 40 minutes at room temperature. If the linkage of the ligand to the resin is intact, the functional groups in the ligand and of the tag are liberated in their entirety. Rinsing is then carried out as follows:

4x 45 seconds with DCE
4x 45 seconds with isopropanol
4x 45 seconds with isopropanol:H_2O = 1:1 (v:v)
5x 45 seconds with H_2O.

Example 8: Marking of thrombin with fluorescein

isothiocyanate is added to a solution of human thrombin (0.53 mg in 100 µl of borate buffer pH 8) (10 µl of a solution of 2.9 mg in 290 µl of DMSO). The reaction is continued for 60 minutes at room temperature and the reaction mixture is worked up over a gel chromatography column (Sephadex®-G25, 15x0,5 cm) (elution with 1M NaCl).

20 µl of the fluorescein-thrombin fraction (c. 3 % of the fraction volume) are diluted to 500 µl and measured photometrically at 495 nm. This gives a fluorescein concentration of c. 2 µm. The fluorescein/thrombin ratio is thus about 1:1.

Example 9: Identification of thrombin-binding ligands in heterogeneous phase

About 500 beads of the resin of Example 7 are incubated in a 2.2 µm solution of

fluorescein-marked thrombin of Example 8 for c. 10 minutes at room temperature. After rinsing for 3x1 minute with a solution of:

68 mg	of imidazole
876 mg	of NaCl
147 mg	of $CaCl_2x2H_2O$
3.35 g	of PEG (3350)
ad. 100 ml of H_2O	

the beads are examined visually in longwave light (blue light at c. 470 nm) for inherent fluorescence. Clearly fluorescing particles are sorted and rinsed

5x 3 minutes with formamide and
10x with H_2O

and subsequently dried.

Example 10: Assay for thrombin inhibition of the ligands of individual beads in liquid phase

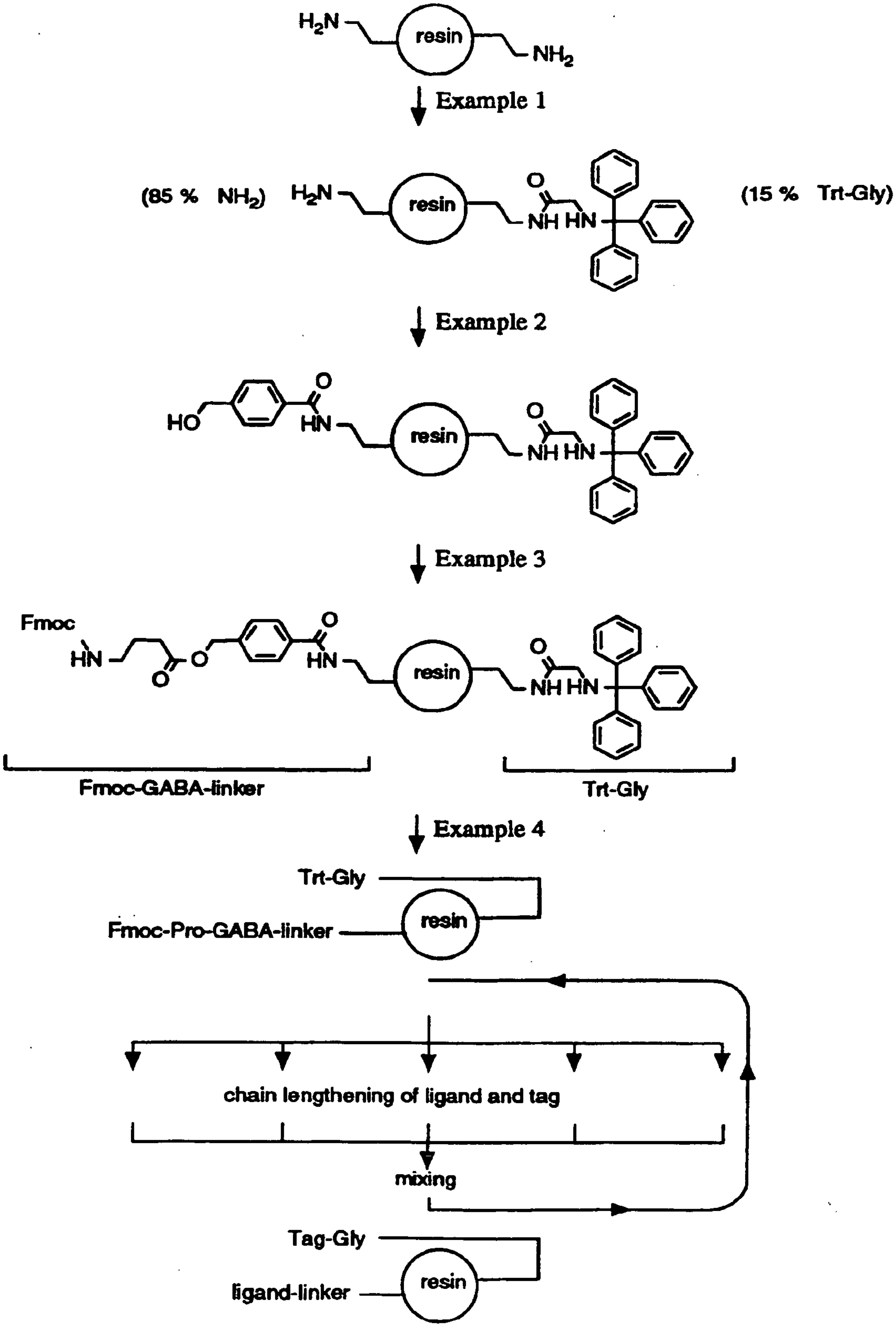
The beads sorted in Example 9 are kept in a saturated solution of ammonia in THF for 24 h at room temperature. Afterwards they are distributed individually into the numbered cavities of a microtitration plate with filter plates (e.g. a Millipore MultiScreen DV96®; pore size 0.65 µm). The ligands can be rinsed with a mixture of tetrahydrofuran/water into a second numbered microtitration plate, where they are assayed direct in a thrombin inhibition assay. Per cavity, $2.3x10^{-2}$ NIH units of thrombin and 9.3 µg of chromogenic substrate S-2302 (D-Pro-Phe-Arg-p-nitroanilide 2HCl; ex Chromogenix) are added in a final volume of 150 µl. The color development over a period of c. 75 minutes is measured with the aid of a Multiwell-Platereader at a wavelength of 405 nm, and the corresponding extinction values are plotted as a function of the time. Cross-comparisons of the gradients of diagrams of different inhibitors act as basis for identifying ligands having inhibitor activity.

The composition of two such identified thrombin-inhibitors is identified from the tag. This is done by screening each of the tags of the beads belonging to each ligand separated by solid-phase protein sequencing (Edman degradation) in an automated sequencing apparatus (ABI 477 available from Applied Biosystems), and identifying the composition of the two ligands from the code table as

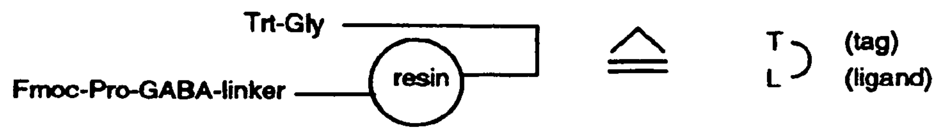
D-Phe-D-Pro-Arg-Pro-GABA
and
D-Phe-Pro-D-Arg-Pro-GABA

Molecular weight (MALDI-MS $M+H^+$) in each case 600.7

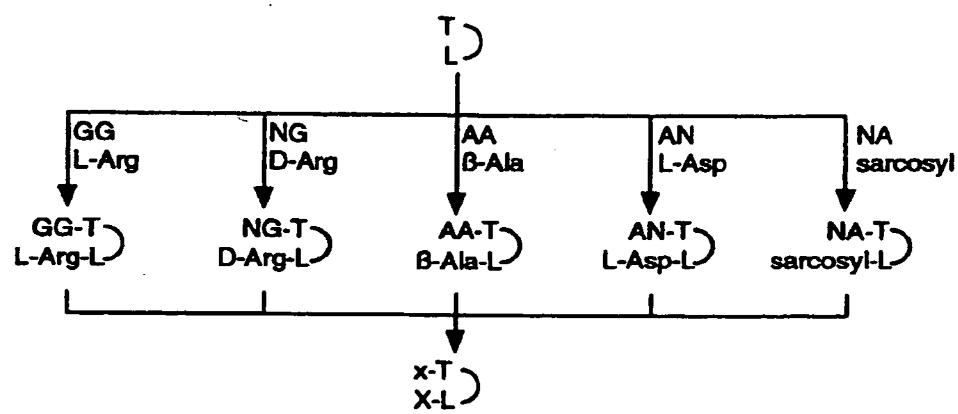
Reaction scheme 1:



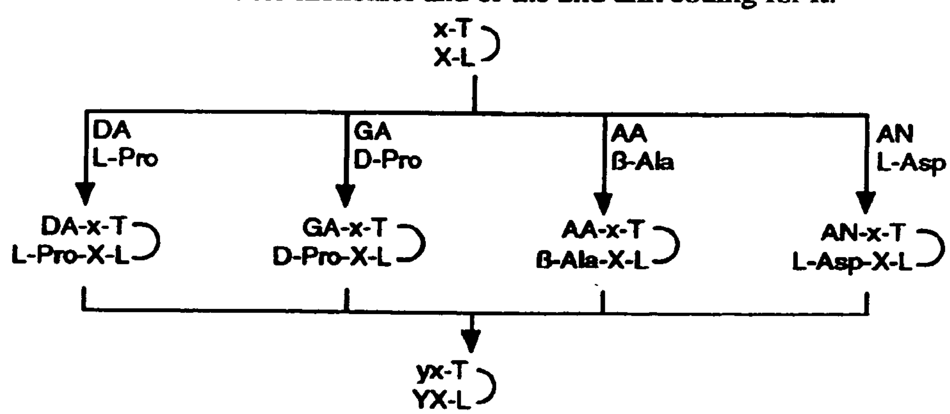
Reaction scheme 2:



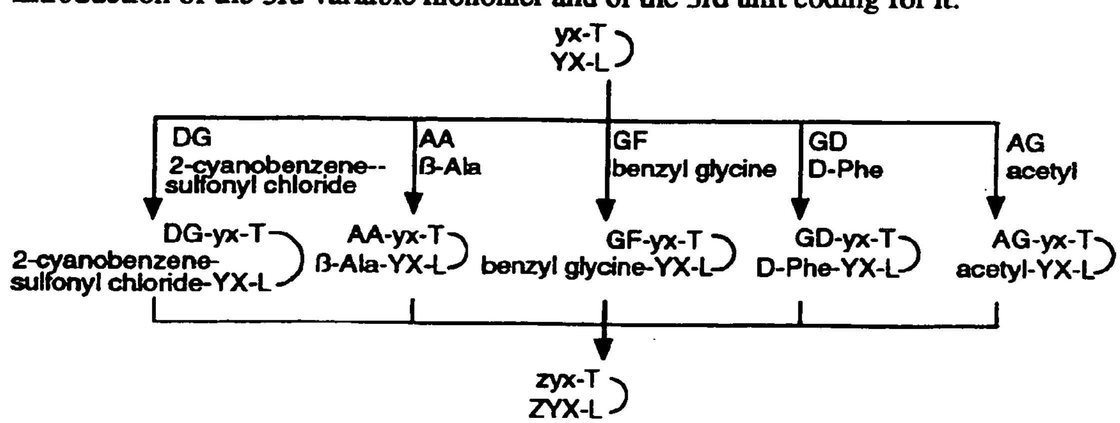
Introduction of the 1st variable monomer into the ligand and of the unit coding for it into the tag:



Introduction of the 2nd variable monomer and of the 2nd unit coding for it:



Introduction of the 3rd variable monomer and of the 3rd unit coding for it:



What is claimed is:

1. A library comprising a plurality of different units each consisting of a solid or semisolid carrier (beads), a synthetic oligomer (ligand) and an identification structure (tag) by means of which the monomers of the ligand can be indentified, wherein:

a) each carrier unit carries only one type of ligand,
b) ligand and tag are attached to the same carrier at different positions,
c) the ligand, irrespective of the tag and without altering its information content, can be separated from the carrier,
d) the tag is a sequenceable polypeptide, and
e) to synthesise the tag or ligand a protective group is used that can be removed under mild acid conditions.

2. A library according to claim 1, wherein the protective group which is removable under mild acidic conditions is at least 10x more labile than the dimethyldimethoxybenzyloxy-carbonyl protective group.

3. A library according to claim 1, wherein the protective group which is removable under mild acidic conditions is a group of the trityl type.

4. A library according to claim 1, wherein the ligands contain monomers which, in the amounts in which they are obtained on a carrier unit, cannot be clearly identified by known and/or automated procedures.

5. A library according to claim 1, wherein the ligand is attached to the carrier through a cleavable linker.

6. A library according to claim 1, wherein the ligand is attached to the carrier through a linker which is cleavable under basic, acidic, photolytic, oxidative or reductive conditions.

7. A library according to claim 1, wherein the ligand is attached to the carrier through a linker which is cleavable under basic conditions.

8. A library according to claim 1, wherein the ligand can be removed in several portions from the carrier.

9. A library according to claim 5, wherein the linker is selected from the group consisting of p-hydroxymethylbenzoic acid, 4-hydroxymethylphenylacetic acid, benzhydrylamino, allyl, hydroxycrotonylaminomethyl, 3-nitro-4-hydroxymethylbenzoic acid, p-nitrobenzhydrylamine, 4-[4,4'-bis(methylsulfinyl)-2-oxybenzhydrylamino]butyric acid and disulfide linkers.

10. A library according to claim 5, wherein the linker is 4-hydroxymethylbenzoic acid.

11. A library according to claim 1, wherein each monomer of the ligand is coded by more than one amino acid.

12. A library according to claim 1, wherein the tag is provided with a constant starting sequence.

13. A library according to claim 1, wherein the tag is provided with a constant end sequence.

14. A library according to claim 1, wherein the ligand is provided with a constant starting sequence.

15. A library according to claim 1, wherein the solid or semisolid carrier is a polymeric resin.

16. A library according to claim 1, wherein the polymeric resin contains free amino groups.

17. A library according to claim 1, wherein the ratio of ligand to tag on a carrier is greater than 1.

18. A library according to claim 1, wherein the ratio of ligand to tag on a carrier is from 2 to 100.

19. A library according to claim 1, wherein the ratio of ligand to tag on a carrier is from 4 to 10.

20. A process for the preparation of a library as claimed in claim 1, which comprises

synthesising ligands and tags by an orthogonal and alternating synthesis.

21. A process for the preparation of a library according to claim 20, which comprises the steps

a) attaching the first unit of the tag and the first monomer of the ligand or the building block thereof or a linker for each to the solid or semi-solid carrier;
b) optionally attaching further invariable monomers to the ligand or further non-variable coding units to the tag;
c) dividing the solid or semisolid carrier into portions for the variable monomers of the ligand;
d) carrying out in each portion separately, in codable sequence, further modifications at the ligand or attaching another of the variable monomers possible at this site of the ligand as well as the unit of the tag coding for this step;
e) mixing the portions;
f) repeating steps b) to e) until the variable part of the ligands is completely synthesised; and
g) optionally attaching one or more than one further non-variable monomer to the ligand or further non-coding units to the tag;

using for the synthesis of tag or ligand a protective group which can be removed under mild acidic conditions.

22. A process according to claim 20, wherein orthogonal protective groups are used for synthesising the ligand and the tag.

23. A process according to claim 20, wherein two groups of protective groups are used for synthesising the ligand and the tag, the first group being removable under mild acidic and the other group under basic conditions, by the action of light, or under oxidative or reductive conditions.

24. A process according to claim 20, wherein two groups of transient protective groups are used for synthesising the ligand and the tag, the first group being removable under mild acidic and the other group under basic conditions.

25. A process according to claim 21, wherein one of the protective groups is of the trityl type (Trt).

26. A process according to claim 21, wherein protective groups of the fluorenylmethoxy-carbonyl type (Fmoc) and of the trityl type (Trt) are used.

27. A process according to claim 19, wherein the synthesis of the ligand is carried out using Fmoc protective groups.

28. A process according to claim 19, wherein the synthesis of the tag is carried out using trityl protective groups.

29. A process according to claim 19, wherein the reactive side-chains of the monomers of the ligand and of the tag which shall not participate in the reactions are protected by a third group of protective groups.

30. A process according to claim 19, wherein the reactive side-chains of the monomers of the ligand or of the tag which shall not participate in the reactions are protected by pentamethylchromanesulfonyl (PMC), Mtr or protective groups of the tert-butyl type.

31. A process for detecting structures, which comprises treating a library as claimed in claim 1 with the acceptor to be investigated and isolating the components of the library that bind the acceptor.

32. A process according to claim 31, wherein the acceptor is a receptor, a transport protein, an antibody, an enzyme or a fragment thereof.

33. A process according to claim 31, wherein the acceptor carries an identifiable group.

34. A process according to claim 33, wherein the identifiable group is a fluorescing, chemoluminescing or radioactive group, avidine, biotine, a reporter enzyme or an immunologically detectable group.

35. A process according to claim 33, wherein the identifiable group is a fluorescing group.

36. A process according to claim 31, wherein the library as claimed in claim 1 is mixed with an acceptor that carries a fluorescing group, washed, and the components of the library that show fluorescence are isolated.

37. A process according to claim 31, wherein the ligands are separated wholly or partially from the isolated carriers and again subjected to an assay.

38. A process according to claim 37, wherein the further assay cannot be carried out with the ligands bound to the solid or semisolid carrier.

39. A process according to claim 37, wherein the further assay is a binding study, NMR or MS.

40. A process according to claim 37, wherein the ligands are separated from the isolated carriers by treatment with a readily volatile or gaseous agent.

41. A process according to claim 37, wherein the ligands are separated from the isolated carriers by treatment with an acid or an alkali.

42. A process according to claim 37, wherein the ligands are separated from the isolated carriers by treatment with an alkali.

43. A process according to claim 37, wherein the ligands are separated from the isolated carriers by treatment with gaseous ammonia, liquid ammonia or a solution of ammonia/THF.

44. A process according to claim 37, wherein a first portion of the ligands is separated from the isolated carriers by treatment with gaseous ammonia, and a second portion by treatment with a solution of ammonia/THF or liquid ammonia.

45. The compound of formula D-Phe-D-Pro-Arg-Pro-GABA or D-Phe-Pro-D-Arg-Pro-GABA, or a pharmaceutically acceptable salt thereof, for inhibiting thrombin.

46. A pharmaceutical composition comprising the compound of formula D-Phe-D-Pro-Arg-Pro-GABA, D-Phe-Pro-D-Arg-Pro-GABA, or a pharmaceutically acceptable salt thereof, and further optional excipients.

INTERNATIONAL SEARCH REPORT

Intern[illegible]al Application No
PCT/EP 94/03936

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68 C07K1/04 C07K17/00 G01N33/86 //G01N33/543, C12Q1/68,G01N33/566,G01N33/58

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PEPTIDE RESEARCH, vol.6, no.3, 1993, NATICK, MASS pages 161 - 170 NIKOLAIEV ET AL. 'Peptide-encoding for structure determination of nonsequenceable polymers within libraries synthesized and tested on solid-phase supports' see the whole document ---	1-46
A	EP,A,0 498 508 (VAN NISPEN) 12 August 1992 see examples ---	45,46

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

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Date of the actual completion of the international search	Date of mailing of the international search report
16 February 1995	27.04.95

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Ceder, O

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,93 06121 (AFFYMAX TECHNOLOGIES) 1 April 1993 see page 3, line 34 - page 5, line 3 see page 16, line 16 - line 24 see page 19, line 8 - line 15 see page 20, line 35 - page 21, line 2 see figures 1,8	1,4-6
A		1-46
Y	EP,A,0 331 184 (THE BOARD OF TRUSTEES OF THE UNIVERSITY OF ALABAMA FOR ITS DIVISION ..) 6 September 1989 see abstract	1,4-6
A	WO,A,92 00091 (BIOLIGAND INC.) 9 January 1992 see page 6, line 1 - page 7, line 20 see page 27, line 22 - page 29, line 4	1,5-10
A	NATURE, vol.354, 7 November 1991 pages 82 - 84 LAM ET AL. 'A new type of synthetic peptide library for identifying ligand-binding activity' cited in the application see page 82; figure 1	1
A	NATURE, vol.354, 7 November 1991 pages 84 - 86 HOUGHTEN ET AL. 'Generation and use of synthetic peptide combinatorial libraries ....' cited in the application see figure 1	1

INTERNATIONAL SEARCH REPORT

Int. ...ional Application No
PCT/EP 94/03936

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		WO-A- 9213877	20-08-92
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(71)出願人　591108237
明治電機工業株式会社
愛知県名古屋市中村区亀島2丁目13番8号

(72)発明者　高橋　利匡
名古屋市中村区亀島二丁目13番8号　明治電機工業株式会社内

(72)発明者　伊藤　敏昭
名古屋市中村区亀島二丁目13番8号　明治電機工業株式会社内

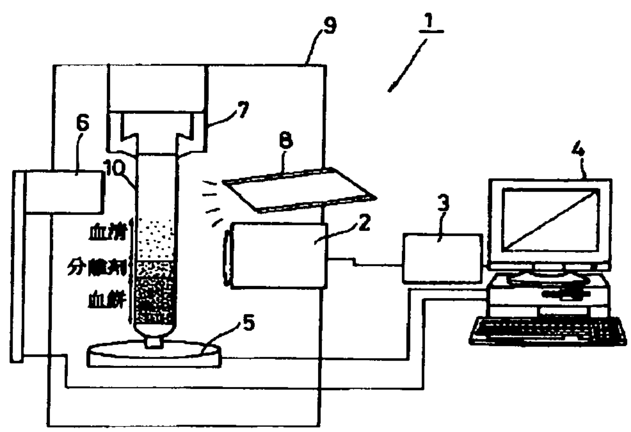
(74)代理人　弁理士　千田　稔

(54)【発明の名称】　採血試験管における血清量測定装置

(57)【要約】

【課題】　採血試験管内の血清量、血餅又は分離剤と血清との境界位置を正確にかつ迅速に測定する装置を安価に提供し、採血試験管から直接分注することを可能にすることを課題とする。

【解決手段】　カラーCCDカメラ2を通して撮影された採血試験管10の撮像情報から、パーソナルコンピュータ4にて画像処理を行ってRGB濃淡情報、色度情報、彩度情報を求め、彩度情報から血液成分のうち血清部分と他の部分との境界線位置を求め、更に、境界情報から採取可能な血清の量を計算することにより、採血試験管から無駄なく血清を採取可能にした。



【特許請求の範囲】

【請求項1】　採血試験管をカラー撮影して、該採血試験管のカラー撮像情報を得る手段と、
前記カラー撮像情報からカラー撮像の各画素においての赤、青、緑の濃淡情報を求める手段と、
前記濃淡情報から明度情報を取り除いて色度情報を求める手段と、
前記色度情報から彩度情報を求める手段と、
前記彩度情報から血液成分のうち血清部分と他の部分との境界線位置を求める手段と、
前記境界情報から採取可能な血清の量を計算する手段と、を含んで構成されたことを特徴とする採血試験管における血清量測定装置。

【請求項2】　前記採血試験管表面の検体IDを示すバーコードラベルが貼られていない位置が撮影方向を向くように、採血試験管をセットする手段を含んで構成されたことを特徴とする請求項1記載の採血試験管における血清量測定装置。

【請求項3】　前記採血試験管をセットする手段は、
前記採血試験管を回転する回転手段と、
前記バーコードラベル位置を検出する光沢度センサと、
前記光沢度センサからの検出信号に基づいて前記回転手段を制御する制御手段と、を含んで構成されたことを特徴とする請求項2記載の採血試験管における血清量測定装置。

【請求項4】　前記彩度により求めた境界線位置近傍の画素の前後所定ドットに対して赤、青、緑各成分の画素の各ドットでの変化量を求め、変化量が最大となる位置を境界線位置とする手段を含んで構成されたことを特徴とする請求項1～3のうちいずれか1つに記載の採血試験管における血清量測定装置。

【請求項5】　前記撮影方向から見た採血試験管の後方に、採血試験管に略密着させて白系統の部材を置いて採血試験管を撮影するようにしたことを特徴とする請求項1～4のうちいずれか1つに記載の採血試験管における血清量測定装置。

【発明の詳細な説明】

【0001】

【発明の属する技術分野】本発明は、採血試験管における血清量測定装置に関し、詳しくは、例えば、分注器等により、遠心分離をかけた採血試験管から自動化学分析に必要な血清を採取するに際して、血清部分の量を予め測定する血清量測定装置に関する。

【0002】

【従来の技術】採血試験管としての真空採血管に遠心分離をかけると、該採血管内において、血清部と血餅部の2層に分離され、分離剤を入れると、血清と分離剤と血餅の3層に分離される。

【0003】このように各成分が分離された真空採血管からは、自動機により、化学分析に必要な血清部分のみを抜き取り、分注を行う。従来では、自動機により分注を行う場合は、真空採血管から別に用意したサンプル容器に血清部のみを移した後に、分注を行う方法が採られていたが、近年では、患者から採血する際に用いた真空採血管自体をサンプル容器として用い、この真空採血管から直接血清部分を採取することが多くなっている。この場合、真空採血管の底部には血餅が沈澱し、分離剤を用いた場合は、この分離剤が血清と血餅との間に残存するようになり、これにより血清と血餅とが真空採血管内において分離される。

【0004】

【発明が解決しようとする課題】しかしながら、上記のように真空採血管から直接的に分注を行う場合、分析に必要な血清のみを所定量サンプリングするのが難しいという問題点がある。例えば、分注の際、血清量が充分確保できていない採血管の場合、分注ノズルが血餅又は分離剤の深さまで到達してしまうことになり、分析に必要な血清以外の成分まで吸引してしまうため、前記分注ノズルを詰まらせる虞がある。

【0005】このため、真空採血管から別に用意したサンプル容器に血清部分のみを移した後に、分注を行うという以前の方法を採らざるを得ない場合がある。このような問題を解決するには、真空採血管から直接分注を行う場合に、遠心分離がかけられた真空採血管内の血清部分の容量を分注前に正確に計測することができれば良く、これが実現すれば、限られた血清量を必要な分析・検査の優先順位の順に割り当て、採血量が少ない患者の血清を有効に利用することが可能となる。

【0006】このような考え方から、従来では、採血試験管内の血清量を測定する方法が次のように提案されている。例えば、採血試験管内に各成分の境界を検出するセンサ（界面検知センサ）を挿入し、血餅又は分離剤の位置を検出する方法が提案されている。この場合のセンサの種類は種々提案されており、例えば特開昭53－71897号公報には、超音波の送受信器を用いた技術が開示され、又、特開昭53－116190号公報等には、光ファイバを用いた技術が開示されている。

【0007】又、電極を挿入し、抵抗値差やインピーダンス差を用いた技術も提案されている。以上のセンサを用いて、血餅又は分離剤の位置を検出する方法では、血清内にセンサを挿入する必要があり、特に、分析に使用する場合は、前サンプルの血清が後のサンプルに混入することを防止するために、1回の採取毎にセンサを充分に洗浄し、又、洗浄後の混入を防止するために洗浄後の乾燥の必要も生じる。又、装置の保守の際等にセンサに触れると、患者の血液からの感染の可能性もあり、危険である。

【0008】又、かかる従来の測定方法では、設備の規模が大掛かりで、設備投資に多大な費用が掛かると共に、採血試験管1本にかかる界面位置検出時間が長く掛

かるという問題点もある。

【0009】更に、試験管を透過する光により、各成分の境界を検出する方法が提案されている。例えば、試験管の外の一方の光源からの光を、試験管を透過させ受光部で受け取り、透過光量変化や光の波長により透過率が異なる特性を求める等により境界を認識する方法が幾つか提案されている。（特開平２－４０５３９、特開平２－３８９６８号公報及び特開平１－４４４６４号公報参照）。

【0010】ところで、真空採血管の表面には、検体ＩＤを示すバーコードラベルを設けるようにした検体ＩＤ方式が採用され、これによって、検体取り扱いミスの防止及び測定の合理化等が図られているが、上記の方法を真空採血管での血清量測定に適用するためには、真空採血管の表面に貼られた検体ＩＤを示すバーコードラベルを取り除くか、バーコードラベルの貼られていない位置での透過検出のみに限定する必要があり、真空採血管からの血清量測定の方法として適さない。

【0011】そこで、本発明は上記に鑑みてなされたものであり、血清内にセンサを挿入することなく、かつ例えば採血試験管の表面に検体ＩＤを示すバーコードラベルが貼られた状態であっても、血清量、血餅又は分離剤と血清との境界位置を正確にかつ迅速に測定する装置を安価に提供し、採血試験管から直接分注することを可能にすることを課題とする。

【0012】

【課題を解決するための手段】このため、請求項１に係る発明は、採血試験管をカラー撮影して、該採血試験管のカラー撮像情報を得る手段と、前記カラー撮像情報からカラー撮像の各画素においての赤、青、緑の濃淡情報を求める手段と、前記濃淡情報から明度情報を取り除いて色度情報を求める手段と、前記色度情報から彩度情報を求める手段と、前記彩度情報から血液成分のうち血清部分と他の部分との境界線位置を求める手段と、前記境界情報から採取可能な血清の量を計算する手段と、を含んで構成した。

【0013】請求項２記載の発明は、前記採血試験管表面の検体ＩＤを示すバーコードラベルが貼られていない位置が撮影方向を向くように、採血試験管をセットする手段を含んで構成した。

【0014】請求項３記載の発明は、前記採血試験管をセットする手段は、前記採血試験管を回転する回転手段と、前記バーコードラベル位置を検出する光沢度センサと、前記光沢度センサからの検出信号に基づいて前記回転手段を制御する制御手段と、を含んで構成した。

【0015】請求項４記載の発明は、前記彩度により求めた境界線位置近傍の画素の前後所定ドットに対して赤、青、緑各成分の画素の各ドットでの変化量を求め、変化量が最大となる位置を境界線位置とする手段を含んで構成した。

【0016】請求項５記載の発明は、前記撮影方向から見た採血試験管の後方に、採血試験管に略密着させて白系統の部材を置いて採血試験管を撮影するようにした。

【0017】

【発明の実施の形態】以下、本発明の実施形態を図面に基づいて詳述する。先ず、本発明の原理について説明する。血液が遠心分離法によって各成分毎に分離された場合、各成分は夫々独自の色を呈する。この色の相違をカラー画像から取り込んだ赤、緑、青（ＲＧＢ）の濃淡情報から認識することによって、血液成分のうち血清部分と他の部分との境界を認識し、これから血清量を求める。

【0018】本発明者らは遠心分離後の採血試験管のカラー画像を分析した。この結果、ＲＧＢの濃淡情報から明度情報を取り除いた色度情報（ＲＧＢの平均値に対するＲＧＢ各成分の混入比率）を求められ、この色度情報から求めた彩度値が血清部分で高い値を示すことが確認できた。

【0019】これは、血清部分は透明度が高くて、鮮やかに画像として見える一方、血餅は黒っぽい色の固形物であり、画像上鮮やかさが低いことによる。又、分離剤も乳白色を呈し、血清部分に比べると、明らかに鮮やかさが低く見えるためである。これらのことは、血清の色が正常な血液が示す黄色のときだけでなく、赤色に近い血清の場合等も当てはまり、殆ど全ての採血管において、彩度の高い領域を認識することにより、血清部分の領域を計算できることになる。

【0020】図１は、上述のような原理に基づく、本発明の血清量測定装置の一実施形態の具体的構成を示す図、図２は、この装置の制御内容を説明するフローチャートである。先ず、図１において、採血試験管の血清量測定装置１は、カラーＣＣＤカメラ（以下、単にカメラと言う）２と、ビデオ入力ボード３と、パーソナルコンピュータ４と、試験管回転用モータ（以下、単にモータと言う）５と、光沢度センサ（バーコードラベル位置検出用）６と、試験管チャック７と、画像撮影用照明８と、から構成されていおり、カメラ２、モータ５、光沢度センサ６、試験管チャック７及び照明８は、夫々測定暗箱９内に配設されている。

【0021】ここで、前記カメラ２は、採血試験管１０をカラー撮影して、該採血試験管１０のカラー撮像情報を得る手段を構成する。又、前記パーソナルコンピュータ４は、前記カラー撮像情報からカラー撮像の各画素においての赤、青、緑（ＲＧＢ）の濃淡情報を求める手段、前記ＲＧＢ濃淡情報から明度情報を取り除いて色度情報を求める手段、前記色度情報から彩度情報を求める手段、前記彩度情報から血液成分のうち血清部分と他の部分との境界線位置を求める手段、前記境界情報から採取可能な血清の量を計算する手段、前記彩度により求めた境界線位置近傍の画素の前後所定ドットに対してＲＧ

Ｂ各成分の画素の各ドットでの変化量を求め、変化量が最大となる位置を境界線位置とする手段の各手段として機能をソフトウェア的に装備している。

【００２２】又、前記モータ５、光沢度センサ６及びパーソナルコンピュータ４は、採血試験管１０を回転させて該採血試験管１０表面の検体ＩＤを示すバーコードラベルが貼られていない位置が撮影方向を向くように、採血試験管１０をセットする手段を構成しており、パーソナルコンピュータ４は、光沢度センサ６からの検出信号に基づいて回転手段としての前記モータ５を制御する制御手段としての機能をソフトウェア的に装備している。

【００２３】かかる血清量測定装置１を用いた血清部分の量測定処理は次のように行う。即ち、採血試験管１０をカメラ２の前に置き、モータ５によって採血試験管１０を回転させてカメラ２の正面に検体ＩＤを示すバーコードラベルが貼られていない位置がくるようにする。

【００２４】この場合、バーコードラベルの貼り位置を検出することによって、逆にバーコードラベルが貼られていない位置を知るが、これは一般に用いられている方法であり、前記光沢度センサ６により容易に行うことができる。カメラ２の前にバーコードラベルが貼られていない位置の採血試験管１０が写っている時点で、採血試験管１０のカラー映像をビデオ入力ボードに取り込む。

【００２５】ビデオ入力ボード３の画像をビットマップ形式でパーソナルコンピュータ４に取込み、採血試験管画像の垂直方向の中心線から左右各８ドット各画素を認識用に抽出し、各画素においてのＲＧＢの濃淡情報に変換する。抽出する画素数は、本実施形態では左右８ドットを採用したが、認識計算の一例として採用したもので、本発明自体が左右８ドットに限定されるものではない。かかるＲＧＢの濃淡情報から、血清部分の液面位置及び血餅又は分離剤と血清部分との境界を認識計算する。

【００２６】次に、図２のフローチャートを参照して、上記の血清部分の量測定処理の詳細を、パーソナルコンピュータ４の制御内容に基づいて説明する。

【００２７】先ずステップ１（図では、Ｓ１と略記する。以下同様）においては、光沢度センサ６を用いて採血試験管の位置決めを行う。即ち、図３に示す認識領域の画像がバーコードラベル１１に重なり合うことなく取り込めるように、光沢度センサ６を用いてバーコードラベルのない位置を制御側に指令する。

【００２８】ステップ２においては、カメラ２で採血試験管１０の撮像情報を得る。即ち、パーソナルコンピュータ４は、光沢度センサ６の制御信号を受け取ったならば、カメラ２で採血試験管撮像情報をキャプチャーする。

【００２９】ステップ３においては、採血試験管情報をカメラ２からＮＴＳＣビデオ入力信号としてビデオ入力ボード３に送信する。

【００３０】ステップ４では、ビデオ入力ボード３からの情報を周知のビットマップ形式に変換する。

【００３１】ステップ５では、ビットマップフォーマット情報から各画素においてのＲＧＢ濃淡情報に変換する。

【００３２】ステップ６では、ＲＧＢ濃淡情報から、明度情報を取り除いた色度情報（ｒ，ｇ，ｂ）にする。

即ち、$r = R / (R + G + B)$

$g = G / (R + G + B)$

$b = G / (R + G + B)$

$r + g + b = 1$ 色度平面

とする。

【００３３】ステップ７では、彩度情報を求める。即ち、色度情報（ｒ，ｇ，ｂ）は、図４（Ａ）に示すように、同一平面上の点（$r + g + b = 1$ 色度平面）の集合となり、同図に示すような平面上の点から彩度を求めるようにする。彩度は、図４（Ｂ）において、ＷＰ／ＷＱの比で表す（参考文献 画像処理応用技術 工業調査会）。この場合、図４（Ｂ）の点Ｗは、正三角形の重心で無彩色を表す。点Ｐは、線分ＷＰの延長上の点と平面上の点 $r + g + b = 1$（$r \geqq 0$，$g \geqq 0$，$b \geqq 0$）の交点である。

【００３４】ステップ８では、境界線の認識を行う。即ち、血清部分は、彩度の値が他の部分よりも高いので、その部分を血清と認識する。これを詳述すると、彩度情報の取り込み開始位置から終端までの移動平均を求める。これをグラフに表すと図５のようになる。血清部分が必ず抽出できる規定値を決め、その規定値により決定されるレベルより上方の部分を血清部分として認識する。血清部分として認識した両端の画素の前後１５ドットを認識域として、その部分の $|R_{n+1} - R_n| + |G_{n+1} - G_n| + |B_{n+1} - B_n|$（図６のグラフ参照）の最大値を血清表面又は血清と分離剤の界面位置として検出する。

【００３５】以上の作用によって求められた血清境界情報に、撮影した採血試験管の種別を与えることにより、採血試験管の種別毎に採取可能な血清の容量を計算することができる。

【００３６】尚、かかる彩度情報に基づく血清量の認識では、使用する照明８を採血試験管１０の正面から当てることによって、採血試験管１０の半周以上にわたりバーコードラベル１１が貼られている場合でも、カメラ２の正面からみたとき、バーコードラベル１１の貼られていない隙間が約６ｍｍ以上あれば血清量の認識が可能である。

【００３７】又、彩度情報に基づき血清領域を認識する本構成では、反射光での撮影であり、バーコードラベルに印刷された文字、記号の影響を受けずに認識が可能である。

【００３８】更に、実際には、上記の各処理に加え、ビ

デオ画像から測定物の大きさを割り出すための距離補正処理を行うのが好ましい。かかる距離補正は、試験管画像と共に距離の定まった点（例えば、ＬＥＤ等）を撮影し、常時距離の補正を行うものであり、測定精度を保証するものである。

【００３９】かかる構成によると、カメラ２によるカラー画像から採血試験管１０内の血清容量及び血餅又は分離剤と血清との境界位置を認識する装置を用いることにより、患者から直接血液を採取した採血試験管１０で、バーコードラベルの貼られた状態のままで、血清量を正確に、分注前に得ることができるため、採取した血液のうち採取可能な血清量に応じて、より優先順位の高い分析・検査から血清を割り当てることが可能となり、貴重な血清の有効利用が可能となる。

【００４０】又、血清液面位置及び血餅又は分離剤と血清との境界位置を分注器に知らせることにより、センサを採血試験管１０内に挿入する必要がなく、よってセンサ洗浄等が不要な安全な分注器を容易に製作することができる。

【００４１】更に、かかる測定装置では、設備の規模が小さく、設備投資にかかる費用が少なくて済み、採血試験管１本にかかる界面位置検出時間も短いという利点がある。

【００４２】尚、上記の構成において、使用する採血試験管の種別は、取り込んだカラー画像から認識することも可能である。又、カメラ２から見た採血試験管１０の後方に、採血試験管１０に略密着させた白系統の部材、例えば白又は淡いグレーの紙又はプラスチックの物体を置くと、採血試験管画像でバーコードラベルの貼られた部分とバーコードラベルの貼られていない部分での血清画像の明度、彩度、色相の差が殆どない画像を得ることができ、バーコードラベルの影響を少なくする採血管の撮影とすることができる。

【００４３】

【発明の効果】以上説明したように、請求項１に係る発明によれば、血清量を正確に、分注前に得ることができるため、採取した血液のうち採取可能な血清量に応じて、より優先順位の高い分析・検査から血清を割り当てることが可能となり、貴重な血清の有効利用が可能となると共に、血清液面位置及び血餅又は分離剤と血清との境界位置を分注器に知らせることにより、センサを採血試験管内に挿入する必要がなく、よってセンサ洗浄等が不要な安全な分注器を容易に製作することができ、しかも、設備の規模が小さく、設備投資にかかる費用が少なくて済み、採血試験管１本にかかる界面位置検出時間も短いという利点がある。

【００４４】請求項２に係る発明によると、患者から直接血液を採取した採血試験管で、検体ＩＤを示すバーコードラベルの貼られた状態のままで、血清量を正確に、分注前に得ることができる。

【００４５】請求項３に係る発明によると、バーコードラベルが貼られていない位置を光沢度センサで容易に検出でき、採血試験管の位置を適正に制御できる。

【００４６】請求項４に係る発明によると、彩度情報から血液成分のうち血清部分と他の部分との境界線位置をより正確に識別できる。

【００４７】請求項５に係る発明によると、採血試験管画像でバーコードラベルの貼られた部分とバーコードラベルの貼られていない部分での血清画像の明度、彩度、色相の差が殆どない画像を得ることができ、バーコードラベルの影響を少なくする採血管の撮影とすることができる。

【図面の簡単な説明】

【図１】　本発明の血清量測定装置の一実施形態の具体的構成を示す図

【図２】　同上装置の制御内容を説明するフローチャート

【図３】　認識領域の画像とバーコードラベルとの関係を示す図

【図４】　（Ａ）は色度情報を示す図、（Ｂ）は彩度を示す図

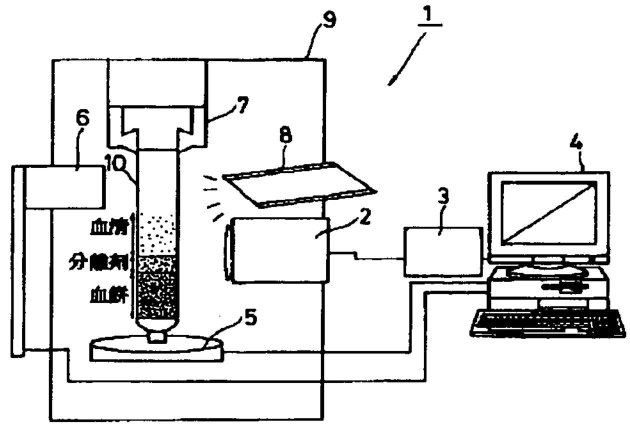
【図５】　彩度情報の取り込み開始位置から終端までの移動平均を表すグラフ

【図６】　$|R_{n+1}-R_n|+|G_{n+1}-G_n|+|B_{n+1}-B_n|$ を表すグラフ

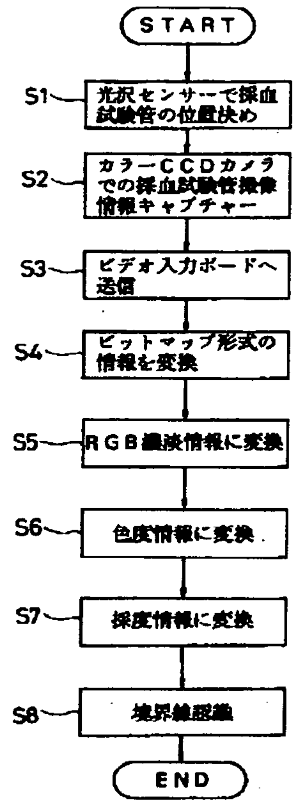
【符号の説明】

１　血清量測定装置
２　カラーＣＣＤカメラ
３　ビデオ入力ボード
４　パーソナルコンピュータ
５　試験管回転用モータ
６　光沢度センサ
１０　採血試験管
１１　バーコードラベル

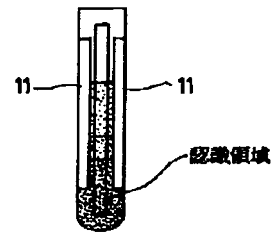
【図1】


1
2
3
4
5
6
7
8
9
10
血清
分離剤
血餅

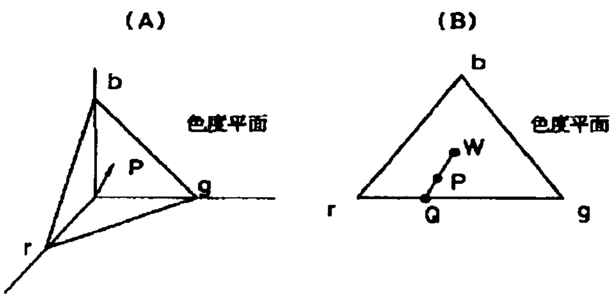
【図2】


START
S1
光沢センサーで採血試験管の位置決め
S2
カラーCCDカメラでの採血試験管撮像情報キャプチャー
S3
ビデオ入力ボードへ送信
S4
ビットマップ形式の情報を変換
S5
RGB濃淡情報に変換
S6
色度情報に変換
S7
彩度情報に変換
S8
境界線認識
END

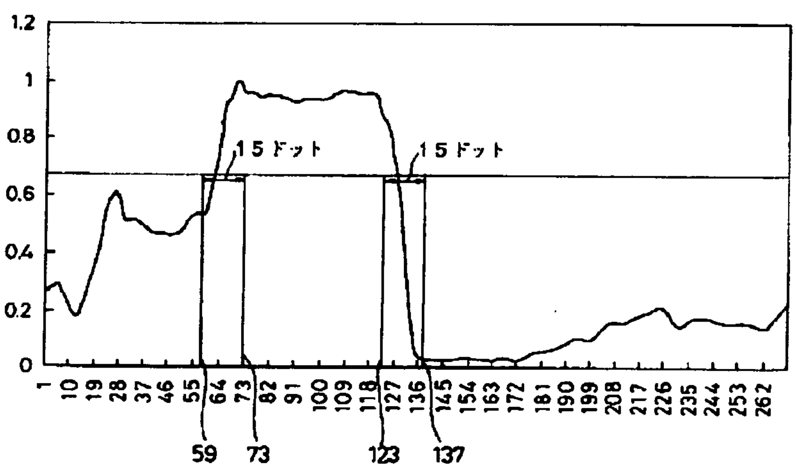
【図3】


11
11
認識領域

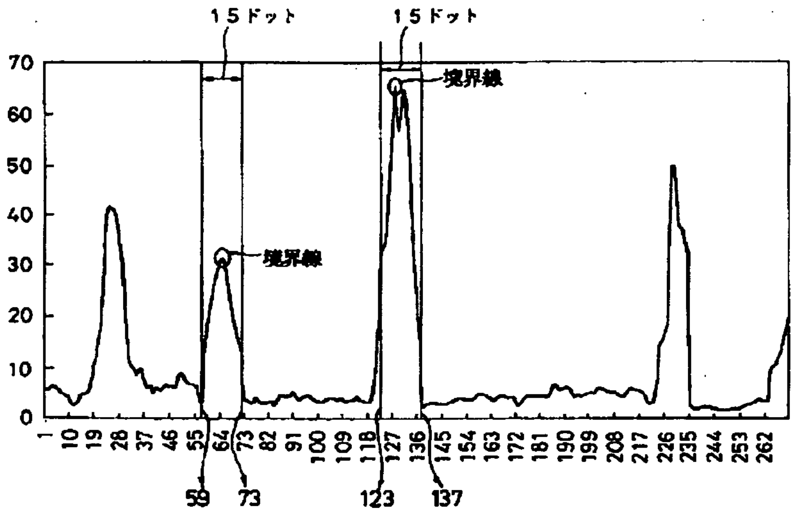
【図4】


(A)
b
色度平面
P
g
r
(B)
b
色度平面
W
P
r
Q
g

【図5】


15ドット
15ドット
59
73
123
137

【図６】



フロントページの続き

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(71)Applicant : MEIJI DENKI KOGYO KK

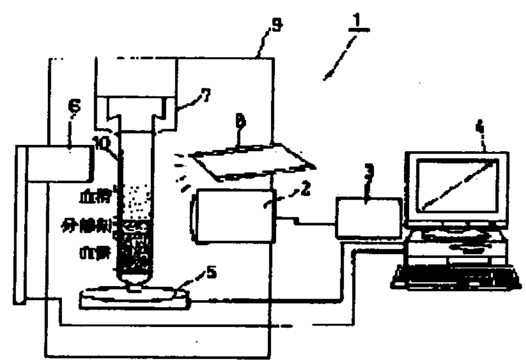
(72)Inventor : TAKAHASHI TOSHITADA
ITO TOSHIAKI

(54) INSTRUMENT FOR MEASURING QUANTITY OF SERUM IN BLOOD-COLLECTING TEST TUBE

(57)Abstract:

PROBLEM TO BE SOLVED: To inexpensively provide an instrument which can accurately and quickly measure the quantity of serum in a blood sample collected in a blood- collecting test tube and the position of the boundary between the blood clot or a separating agent and the serum and, in addition, to make it possible to directly dispense a blood sample from the test tube.

SOLUTION: A personal computer 4 obtains RGB gray level information, chromaticity information, and chroma information by processing the picture information of a blood-collecting test tube 10 taken with a color CCD camera 12 and finds the position of the boundary between the serum part and other parts of a blood sample collected in the tube 10 from the chroma information. In addition, the serum is made to be collected effectively from the test tube 10 by calculating the quantity of a collectable serum from the boundary information.



LEGAL STATUS

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CLAIMS

[Claim(s)]
[Claim 1] A means to carry out color photography of the blood collecting test tube, and to acquire the color image pick-up information on this blood collecting test tube, A means to search for the red in each pixel of a color image pick-up, blue, and green shade information from said color image pick-up information, A means to remove lightness information from said shade information, and to search for chromaticity information, and a means to search for saturation information from said chromaticity information, The amount measuring device of blood serums in the blood collecting test tube characterized by consisting of said saturation information including a means to ask for the borderline location of a blood serum part and other parts among constituents of blood, and a means to calculate the amount of a blood serum extractable from said boundary information.
[Claim 2] The amount measuring device of blood serums in the blood collecting test tube according to claim 1 characterized by being constituted including a means to set a blood collecting test tube so that the location where the bar code label in which the specimen ID of said blood collecting test tube front face is shown is not stuck may turn to bearing of the exposure axis.
[Claim 3] A means to set said blood collecting test tube is the amount measuring device of blood serums in the blood collecting test tube according to claim 2 characterized by being constituted including a revolution means to rotate said blood collecting test tube, the glossiness sensor which detects said bar code label location, and the control means which controls said revolution means based on the detecting signal from said glossiness sensor.
[Claim 4] The amount measuring device [in / among claims 1-3 characterized by being constituted including the means which makes a borderline location the location where the variation in each dot of the pixel of red, blue, and a **** component is calculated from the pixel order predetermined dot near / for which it asked with said saturation / the borderline location, and variation serves as max / the blood collecting test tube of any one publication] of blood serums.
[Claim 5] The amount measuring device [in / among claims 1-4 characterized by making a blood collecting test tube carry out abbreviation adhesion, placing the member of a white system behind the blood collecting test tube seen from said bearing of the exposure axis, and photoing a blood collecting test tube / the blood collecting test tube of any one publication] of blood serums.

[Translation done.]

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1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DETAILED DESCRIPTION

[Detailed Description of the Invention]
[0001]
[Field of the Invention] About the amount measuring device of blood serums in a blood collecting test tube, in detail, this invention is faced extracting a blood serum required for automation study analysis with a distributive-pouring vessel etc. from the blood collecting test tube to which centrifugal separation was applied, and relates to the amount measuring device of blood serums which measures the amount of a blood serum part beforehand.
[0002]
[Description of the Prior Art] If centrifugal separation is applied to vacuum blood collecting tubing as a blood collecting test tube, it will separate into two-layer [of the blood serum section and the clot section] in this blood collecting tubing, and if a separating medium is put in, it will separate into three layers, a blood serum, a separating medium, and a clot.
[0003] Thus, from vacuum blood collecting tubing with which each component was separated, it pours distributively by sampling only a blood serum part required for a chemical analysis with an automatic machine. Although the approach of pouring distributively was taken in the former after moving only the blood serum section from vacuum blood collecting tubing to the sample container prepared independently when pouring distributively with an automatic machine, the direct blood serum part is extracted more often from this vacuum blood collecting tubing in recent years, using the vacuum blood collecting tubing itself used when collecting blood from a patient as a sample container. In this case, when a clot precipitates at the pars basilaris ossis occipitalis of vacuum blood collecting tubing and a separating medium is used, this separating medium comes to remain between a blood serum and a clot, and, thereby, a blood serum and a clot are separated in vacuum blood collecting tubing.
[0004]
[Problem(s) to be Solved by the Invention] However, when pouring distributively directly from vacuum blood collecting tubing as mentioned above, there is a trouble that it is difficult to carry out the specified quantity sampling only of the blood serum required for analysis. For example, since a distributive-pouring nozzle will reach to the depth of a clot or a separating medium and draws in to components other than a blood serum required for analysis when the amount of blood serums is not securable enough blood collecting tubing in the case of distributive pouring, there is a possibility of blocking said distributive-pouring nozzle.
[0005] For this reason, after moving only a blood serum part from vacuum blood collecting tubing to the sample container prepared independently, the approach before saying that it pours distributively is taken. If this is realized that what is necessary is just to be able to measure to accuracy before pouring distributively the capacity of the blood serum part in vacuum blood collecting tubing with which centrifugal separation was applied when pouring distributively directly from vacuum blood collecting tubing, in order to solve such a problem, the limited amount of blood serums will be assigned in order of the priority of required analysis and inspection, and the amount of blood collecting will become possible [using few patients' blood serum effectively].
[0006] From such a view, the approach of measuring the amount of blood serums in a blood

collecting test tube is proposed as follows by the former. For example, the sensor (interface detection sensor) which detects the boundary of each component is inserted into a blood collecting test tube, and the method of detecting the location of a clot or a separating medium is proposed. The technique which the technique which various classes of sensor in this case are proposed, for example, used the transceiver machine of a supersonic wave for JP,53-71897,A was indicated, and used the optical fiber for JP,53-116190,A is indicated.
[0007] Moreover, an electrode is inserted and the technique using a resistance difference or an impedance difference is also proposed. By the approach of detecting the location of a clot or a separating medium using the above sensor, the need for the desiccation after washing in order to insert a sensor into a blood serum, to fully wash a sensor for every one extraction in order that the blood serum of a before sample may prevent mixing in a next sample, especially when using it for analysis, and to prevent mixing after washing is also produced. Moreover, if a sensor is touched in the case of maintenance of equipment etc., there is possibility of the infection from a patient's blood and it is dangerous.
[0008] Moreover, by this conventional measuring method, while the magnitude of a facility is large-scale and great costs start plant-and-equipment investment, there is also a trouble that the interface location detection time concerning one blood collecting test tube starts for a long time.
[0009] Furthermore, the method of detecting the boundary of each component is proposed by the light which penetrates a test tube. For example, some methods of recognizing a boundary in quest of the property that make a test tube penetrate and permeability changes the light from one light source besides a test tube with wavelength of reception, the amount change of transmitted lights, or light by the light sensing portion etc. are proposed. (Refer to JP,2-40539,A, JP,2-38968,A, and JP,1-44464,A) .
[0010] By the way, although specimen ID signalling which prepared the bar code label in which Specimen ID is shown in the front face of vacuum blood collecting tubing is adopted and prevention of a specimen handling mistake, rationalization of measurement, etc. are attained by this In order to apply the above-mentioned approach to the amount measurement of blood serums with vacuum blood collecting tubing It is necessary to remove the bar code label in which the specimen ID stuck on the front face of vacuum blood collecting tubing is shown, or to limit only to transparency detection in the location where a bar code label is not stuck, and is not suitable as the approach of the amount measurement of blood serums from vacuum blood collecting tubing.
[0011] Then, even if you are in the condition that the bar code label in which Specimen ID is shown was stuck on the front face of for example, a blood collecting test tube, without making this invention in view of the above, and inserting a sensor into a blood serum, the equipment which measures correctly and promptly the boundary location of the amount of blood serums, a clot or a separating medium, and a blood serum is offered cheaply, and let it be a technical problem to make it possible to annotate by direct from a blood collecting test tube.
[0012]
[Means for Solving the Problem] For this reason, a means for invention concerning claim 1 to carry out color photography of the blood collecting test tube, and to acquire the color image pick-up information on this blood collecting test tube, A means to search for the red in each pixel of a color image pick-up, blue, and green shade information from said color image pick-up information, A means to remove lightness information from said shade information, and to search for chromaticity information, and a means to search for saturation information from said chromaticity information, It constituted from said saturation information including a means to ask for the borderline location of a blood serum part and other parts among constituents of blood, and a means to calculate the amount of a blood serum extractable from said boundary information.
[0013] Invention according to claim 2 was constituted including a means to set a blood collecting test tube so that the location where the bar code label in which the specimen ID of said blood collecting test tube front face is shown is not stuck might turn to bearing of the exposure axis.
[0014] A means by which invention according to claim 3 set said blood collecting test tube was

constituted including a revolution means to rotate said blood collecting test tube, the glossiness sensor which detects said bar code label location, and the control means which controls said revolution means based on the detecting signal from said glossiness sensor.
[0015] Invention according to claim 4 calculated the variation in each dot of the pixel of red, blue, and a **** component from the pixel order predetermined dot near [for which it asked with said saturation] the borderline location, and variation constituted it including the means which makes the location used as max a borderline location.
[0016] Behind the blood collecting test tube seen from said bearing of the exposure axis, a blood collecting test tube is made to carry out abbreviation adhesion, and invention according to claim 5 places the member of a white system, and photoed the blood collecting test tube.
[0017]
[Embodiment of the Invention] Hereafter, the operation gestalt of this invention is explained in full detail based on a drawing. First, the principle of this invention is explained. When blood is separated for every component by the centrifuge method, each component presents an original color, respectively. By recognizing a difference of this color from the red who incorporated from the color picture, green, and the shade information on blue (RGB), the boundary of a blood serum part and other parts is recognized among constituents of blood, and the amount of blood serums is calculated after this.
[0018] this invention persons analyzed the color picture of the blood collecting test tube after centrifugal separation. Consequently, the chromaticity information (mixing ratio of RGB each component to the average of RGB) which removed lightness information from the shade information on RGB could be searched for, and it has checked that the saturation value calculated from this chromaticity information showed a value high in a blood serum part.
[0019] While this looks a blood serum part is highly transparent and vivid as an image, a clot is the solid of a blackish color and image top vividness depends it on a low thing. Moreover, it is because a separating medium also presents opalescence and vividness looks low clearly compared with a blood serum part. Not only when it is the yellow which blood with the normal color of a blood serum shows, but in the blood serum near red etc., it will be applied, and these things can calculate the field of a blood serum part in almost all blood collecting tubing by recognizing the field where saturation is high.
[0020] Drawing showing the concrete configuration of 1 operation gestalt of the amount measuring device of blood serums of this invention based on the above principles in drawing 1 and drawing 2 are the flow charts explaining the content of control of this equipment. In drawing 1 first, the amount measuring device 1 of blood serums of a blood collecting test tube Color CCD camera (only henceforth a camera) 2, and the video input board 3, A personal computer 4 and the motor 5 for a test tube revolution (only henceforth a motor), the glossiness sensor (for bar code label location detection) 6, the test tube chuck 7, and the lighting 8 for image photography -- since -- it is constituted, and gets down and a camera 2, a motor 5, the glossiness sensor 6, the test tube chuck 7, and lighting 8 are arranged in the measurement black box 9, respectively.
[0021] Here, said camera 2 carries out color photography of the blood collecting test tube 10, and constitutes a means to acquire the color image pick-up information on this blood collecting test tube 10. Moreover, red [in / in said personal computer 4 / each pixel of the color image pick-up from said color image pick-up information], Blue, a means to search for shade information [being green (RGB)], a means to remove lightness information from said RGB shade information, and to search for chromaticity information, A means to search for saturation information from said chromaticity information, a means to ask for the borderline location of a blood serum part and other parts among constituents of blood from said saturation information, The variation in each dot of the pixel of RGB each component is calculated from the pixel order predetermined dot near [for which it asked with a means to calculate the amount of a blood serum extractable from said boundary information, and said saturation] the borderline location. Variation has equipped the function by software as each means of the means which makes the location used as max a borderline location.
[0022] Moreover, a means set the blood-collecting test tube 10 constitutes, and a personal

computer 4 has equipped by software the function as a control means which controls said motor 5 as a revolution means based on the detecting signal from the glossiness sensor 6 so that the location where the bar code label in which said motor 5, the glossiness sensor 6, and a personal computer 4 rotate the blood-collecting test tube 10, and the specimen ID of this blood-collecting test tube 10 front face is shown is not stuck may turn to bearing of the exposure axis.

[0023] Amount measurement processing of the blood serum part using this amount measuring device 1 of blood serums is performed as follows. That is, the blood collecting test tube 10 is placed in front of a camera 2, and it is made for the location where the bar code label which is made to rotate the blood collecting test tube 10, and shows Specimen ID to the transverse plane of a camera 2 by the motor 5 is not stuck to come.
[0024] In this case, although the location where the bar code label is not stuck on reverse by a bar code label's sticking and detecting a location is got to know, this is an approach generally used and said glossiness sensor 6 can perform it easily. When the blood collecting test tube 10 of the location where the bar code label is not stuck in front of the camera 2 is reflected, the color image of the blood collecting test tube 10 is incorporated on a video input board.
[0025] The image of the video input board 3 is captured in a personal computer 4 in a bit map format, 8 dot each each [of right and left] pixel is extracted from the center line of the perpendicular direction of a blood collecting test tube image to recognition, and it changes into the shade information on RGB in each pixel. Although 8 dots of right and left were used for the number of pixels to extract with this operation gestalt, it is what was adopted as an example of recognition count, and this invention itself is not limited to 8 dots of right and left. From the shade information on this RGB, recognition count of the boundary of the oil-level location of a blood serum part and a clot or a separating medium, and a blood serum part is carried out.
[0026] Next, with reference to the flow chart of drawing 2 , the detail of amount measurement processing of the above-mentioned blood serum part is explained based on the content of control of a personal computer 4.
[0027] It is step 1 (it is written as S1 by a diagram.) first. the following -- being the same -- it sets and a blood collecting test tube is positioned using the glossiness sensor 6. That is, a control side is ordered the location which does not have a bar code label using the glossiness sensor 6 so that it can incorporate without the image of the recognition field shown in drawing 3 overlapping the bar code label 11.
[0028] In step 2, the image pick-up information on the blood collecting test tube 10 is acquired with a camera 2. That is, if a personal computer 4 receives the control signal of the glossiness sensor 6, it will capture blood collecting test tube image pick-up information with a camera 2.
[0029] In step 3, blood collecting test tube information is transmitted to the video input board 3 as an NTSC video input signal from a camera 2.
[0030] At step 4, the information from the video input board 3 is changed into a well-known bit map format.
[0031] At step 5, it changes into the RGB shade information in each pixel from bit map format information.
[0032] At step 6, it is made the chromaticity information (r, g, b) which removed lightness information from RGB shade information.
Namely, $r=R/(R+G+B)$
$g=G/(R+G+B)$
$b=G/(R+G+B)$
$r+g+b=1$ It considers as a chromaticity flat surface.
[0033] Saturation information is searched for at step 7. That is, as shown in drawing 4 (A), chromaticity information (r, g, b) serves as a set of the point on the same flat surface ($r+g+b=1$ chromaticity flat surface), and asks for saturation from the point on a flat surface as shown in this drawing. Saturation is expressed with the ratio of WP/WQ in drawing 4 (B) (reference image-processing applied-technology Kogyo Chosakai Publishing). In this case, the point W of drawing 4 (B) expresses an achromatic color at the center of gravity of an equilateral triangle. Points P are a point on extension of Segment WP, and an intersection of point $r+g+b=1$ ($r>=0$, $g>=0$, $b>=0$) on

a flat surface.
[0034] A borderline is recognized at step 8. That is, since a blood serum part has the value of saturation higher than other parts, it recognizes the part to be a blood serum. A detailed description of this asks for the moving average from the incorporation starting position of saturation information to termination. If this is expressed with a graph, it will become like drawing 5 . A blood serum part determines the default value which can surely be extracted, and recognizes an upper part as a blood serum part from the level determined with the default value. The maximum of $|R_{n+1}-R_n|+|G_{n+1}-G_n|+|B_{n+1}-B_n|$ (refer to the graph of drawing 6) of the part is detected by making 15 dots into a recognition region around the pixel of the ends recognized as a blood serum part as an interface location of a blood serum front face or a blood serum, and a separating medium.
[0035] The capacity of a blood serum extractable for every classification of a blood collecting test tube is calculable by giving the classification of the photoed blood collecting test tube to the blood serum boundary information searched for according to the above operation.
[0036] In addition, when the rear-spring-supporter bar code label 11 is stuck more than the semicircle of the blood collecting test tube 10 by putting the lighting 8 to be used in the recognition of the amount of blood serums based on this saturation information from the transverse plane of the blood collecting test tube 10 and it sees from the transverse plane of a camera 2, if there is about 6mm or more of clearances where the bar code label 11 is not stuck, recognition of the amount of blood serums is possible.
[0037] Moreover, it can recognize with this configuration which recognizes a blood serum field based on saturation information, without being influenced of the alphabetic character which is photography by the reflected light and was printed by the bar code label, and a notation.
[0038] Furthermore, it is desirable to perform range correction processing for deducing the magnitude of a measurement object from a video image actually in addition to each above-mentioned processing. This range correction photos the points (for example, LED etc.) that distance became settled with the test tube image, always amends distance, and guarantees the accuracy of measurement.
[0039] With the blood collecting test tube 10 which extracted direct blood from the patient by using the equipment which recognizes the boundary location of the blood serum capacity in the blood collecting test tube 10 and a clot or a separating medium, and a blood serum from a color picture with a camera 2 according to this configuration With the condition that the bar code label was stuck, since the amount of blood serums can be obtained before distributive pouring to accuracy, among the extracted blood, according to the extractable amount of blood serums, it becomes possible to assign a blood serum from analysis and inspection with high priority more, and a deployment of a precious blood serum is attained.
[0040] Moreover, it is not necessary to insert a sensor into the blood collecting test tube 10, and, therefore, sensor washing etc. can manufacture an unnecessary safe distributive-pouring machine easily by telling a distributive-pouring machine about the boundary location of a blood serum oil-level location and a clot or a separating medium, and a blood serum.
[0041] Furthermore, in this measuring device, the magnitude of a facility is small, there are few costs concerning plant-and-equipment investment, and they end, and the interface location detection time concerning one blood collecting test tube also has the advantage of being short.
[0042] In addition, in the above-mentioned configuration, the classification of the blood collecting test tube to be used can also be recognized from the incorporated color picture. Moreover, if the paper of the member of the white system which carried out abbreviation adhesion, for example, white, and light gray, or the body of plastics is placed behind the blood collecting test tube 10 seen from the camera 2 at the blood collecting test tube 10 The lightness of the blood serum image in the part on which the bar code label was stuck by the blood collecting test tube image, and the part on which a bar code label is not stuck, saturation, and the image that does not almost have the difference of a hue can be obtained, and it can consider as photography of blood collecting tubing which lessens effect of a bar code label.
[0043]
[Effect of the Invention] Since the amount of blood serums can be obtained before distributive

pouring to accuracy according to invention concerning claim 1 as explained above, While becoming possible to assign a blood serum from analysis and inspection with high priority more and attaining a deployment of a precious blood serum according to the extractable amount of blood serums among the extracted blood By telling a distributive-pouring machine about the boundary location of a blood serum oil-level location and a clot or a separating medium, and a blood serum It is not necessary to insert a sensor into a blood collecting test tube, and therefore sensor washing etc. can manufacture an unnecessary safe distributive-pouring machine easily, moreover, the magnitude of a facility is small, there are few costs concerning plant-and-equipment investment, and they end, and the interface location detection time concerning one blood collecting test tube also has the advantage of being short.
[0044] According to invention concerning claim 2, the amount of blood serums can be obtained from a patient before distributive pouring to accuracy with the condition which shows Specimen ID with the blood collecting test tube which extracted direct blood that the bar code label was stuck.
[0045] According to invention concerning claim 3, a glossiness sensor can detect easily the location where the bar code label is not stuck, and the location of a blood collecting test tube can be controlled proper.
[0046] According to invention concerning claim 4, the borderline location of a blood serum part and other parts is more discriminable from saturation information to accuracy among constituents of blood.
[0047] According to invention concerning claim 5, the lightness of the blood serum image in the part on which the bar code label was stuck by the blood collecting test tube image, and the part on which a bar code label is not stuck, saturation, and the image that does not almost have the difference of a hue can be obtained, and it can consider as photography of blood collecting tubing which lessens effect of a bar code label.

[Translation done.]

TECHNICAL FIELD

[Field of the Invention] About the amount measuring device of blood serums in a blood collecting test tube, in detail, this invention is faced extracting a blood serum required for automation study analysis with a distributive-pouring vessel etc. from the blood collecting test tube to which centrifugal separation was applied, and relates to the amount measuring device of blood serums which measures the amount of a blood serum part beforehand.

[Translation done.]

* NOTICES *

JPO and NCIPI are not responsible for any
damages caused by the use of this translation.

1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

PRIOR ART

[Description of the Prior Art] If centrifugal separation is applied to vacuum blood collecting tubing as a blood collecting test tube, it will separate into two-layer [of the blood serum section and the clot section] in this blood collecting tubing, and if a separating medium is put in, it will separate into three layers, a blood serum, a separating medium, and a clot.
[0003] Thus, from vacuum blood collecting tubing with which each component was separated, it pours distributively by sampling only a blood serum part required for a chemical analysis with an automatic machine. Although the approach of pouring distributively was taken in the former after moving only the blood serum section from vacuum blood collecting tubing to the sample container prepared independently when pouring distributively with an automatic machine, the direct blood serum part is extracted more often from this vacuum blood collecting tubing in recent years, using the vacuum blood collecting tubing itself used when collecting blood from a patient as a sample container. In this case, when a clot precipitates at the pars basilaris ossis occipitalis of vacuum blood collecting tubing and a separating medium is used, this separating medium comes to remain between a blood serum and a clot, and, thereby, a blood serum and a clot are separated in vacuum blood collecting tubing.

[Translation done.]

EFFECT OF THE INVENTION

[Effect of the Invention] Since the amount of blood serums can be obtained before distributive pouring to accuracy according to invention concerning claim 1 as explained above, While becoming possible to assign a blood serum from analysis and inspection with high priority more and attaining a deployment of a precious blood serum according to the extractable amount of blood serums among the extracted blood By telling a distributive-pouring machine about the boundary location of a blood serum oil-level location and a clot or a separating medium, and a blood serum It is not necessary to insert a sensor into a blood collecting test tube, and therefore sensor washing etc. can manufacture an unnecessary safe distributive-pouring machine easily, moreover, the magnitude of a facility is small, there are few costs concerning plant-and-equipment investment, and they end, and the interface location detection time concerning one blood collecting test tube also has the advantage of being short.
[0044] According to invention concerning claim 2, the amount of blood serums can be obtained from a patient before distributive pouring to accuracy with the condition which shows Specimen ID with the blood collecting test tube which extracted direct blood that the bar code label was stuck.
[0045] According to invention concerning claim 3, a glossiness sensor can detect easily the location where the bar code label is not stuck, and the location of a blood collecting test tube can be controlled proper.
[0046] According to invention concerning claim 4, the borderline location of a blood serum part and other parts is more discriminable from saturation information to accuracy among constituents of blood.
[0047] According to invention concerning claim 5, the lightness of the blood serum image in the part on which the bar code label was stuck by the blood collecting test tube image, and the part on which a bar code label is not stuck, saturation, and the image that does not almost have the difference of a hue can be obtained, and it can consider as photography of blood collecting tubing which lessens effect of a bar code label.

[Translation done.]

* NOTICES *

TECHNICAL PROBLEM

[Problem(s) to be Solved by the Invention] However, when pouring distributively directly from vacuum blood collecting tubing as mentioned above, there is a trouble that it is difficult to carry out the specified quantity sampling only of the blood serum required for analysis. For example, since a distributive-pouring nozzle will reach to the depth of a clot or a separating medium and draws in to components other than a blood serum required for analysis when the amount of blood serums is not securable enough blood collecting tubing in the case of distributive pouring, there is a possibility of blocking said distributive-pouring nozzle.
[0005] For this reason, after moving only a blood serum part from vacuum blood collecting tubing to the sample container prepared independently, the approach before saying that it pours distributively is taken. If this is realized that what is necessary is just to be able to measure to accuracy before pouring distributively the capacity of the blood serum part in vacuum blood collecting tubing with which centrifugal separation was applied when pouring distributively directly from vacuum blood collecting tubing, in order to solve such a problem, the limited amount of blood serums will be assigned in order of the priority of required analysis and inspection, and the amount of blood collecting will become possible [using few patients' blood serum effectively].
[0006] From such a view, the approach of measuring the amount of blood serums in a blood collecting test tube is proposed as follows by the former. For example, the sensor (interface detection sensor) which detects the boundary of each component is inserted into a blood collecting test tube, and the method of detecting the location of a clot or a separating medium is proposed. The technique which the technique which various classes of sensor in this case are proposed, for example, used the transceiver machine of a supersonic wave for JP,53-71897,A was indicated, and used the optical fiber for JP,53-116190,A is indicated.
[0007] Moreover, an electrode is inserted and the technique using a resistance difference or an impedance difference is also proposed. By the approach of detecting the location of a clot or a separating medium using the above sensor, the need for the desiccation after washing in order to insert a sensor into a blood serum, to fully wash a sensor for every one extraction in order that the blood serum of a before sample may prevent mixing in a next sample, especially when using it for analysis, and to prevent mixing after washing is also produced. Moreover, if a sensor is touched in the case of maintenance of equipment etc., there is possibility of the infection from a patient's blood and it is dangerous.
[0008] Moreover, by this conventional measuring method, while the magnitude of a facility is large-scale and great costs start plant-and-equipment investment, there is also a trouble that the interface location detection time concerning one blood collecting test tube starts for a long time.
[0009] Furthermore, the method of detecting the boundary of each component is proposed by the light which penetrates a test tube. For example, some methods of recognizing a boundary in quest of the property that make a test tube penetrate and permeability changes the light from one light source besides a test tube with wavelength of reception, the amount change of transmitted lights, or light by the light sensing portion etc. are proposed. (Refer to JP,2-40539,A, JP,2-38968,A, and JP,1-44464,A) .

[0010] By the way, although specimen ID signalling which prepared the bar code label in which Specimen ID is shown in the front face of vacuum blood collecting tubing is adopted and prevention of a specimen handling mistake, rationalization of measurement, etc. are attained by this In order to apply the above-mentioned approach to the amount measurement of blood serums with vacuum blood collecting tubing It is necessary to remove the bar code label in which the specimen ID stuck on the front face of vacuum blood collecting tubing is shown, or to limit only to transparency detection in the location where a bar code label is not stuck, and is not suitable as the approach of the amount measurement of blood serums from vacuum blood collecting tubing.

[0011] Then, even if you are in the condition that the bar code label in which Specimen ID is shown was stuck on the front face of for example, a blood collecting test tube, without making this invention in view of the above, and inserting a sensor into a blood serum, the equipment which measures correctly and promptly the boundary location of the amount of blood serums, a clot or a separating medium, and a blood serum is offered cheaply, and let it be a technical problem to make it possible to annotate by direct from a blood collecting test tube.

[Translation done.]

MEANS

[Means for Solving the Problem] For this reason, a means for invention concerning claim 1 to carry out color photography of the blood collecting test tube, and to acquire the color image pick-up information on this blood collecting test tube, A means to search for the red in each pixel of a color image pick-up, blue, and green shade information from said color image pick-up information, A means to remove lightness information from said shade information, and to search for chromaticity information, and a means to search for saturation information from said chromaticity information, It constituted from said saturation information including a means to ask for the borderline location of a blood serum part and other parts among constituents of blood, and a means to calculate the amount of a blood serum extractable from said boundary information.
[0013] Invention according to claim 2 was constituted including a means to set a blood collecting test tube so that the location where the bar code label in which the specimen ID of said blood collecting test tube front face is shown is not stuck might turn to bearing of the exposure axis.
[0014] A means by which invention according to claim 3 set said blood collecting test tube was constituted including a revolution means to rotate said blood collecting test tube, the glossiness sensor which detects said bar code label location, and the control means which controls said revolution means based on the detecting signal from said glossiness sensor.
[0015] Invention according to claim 4 calculated the variation in each dot of the pixel of red, blue, and a **** component from the pixel order predetermined dot near [for which it asked with said saturation] the borderline location, and variation constituted it including the means which makes the location used as max a borderline location.
[0016] Behind the blood collecting test tube seen from said bearing of the exposure axis, a blood collecting test tube is made to carry out abbreviation adhesion, and invention according to claim 5 places the member of a white system, and photoed the blood collecting test tube.
[0017]
[Embodiment of the Invention] Hereafter, the operation gestalt of this invention is explained in full detail based on a drawing. First, the principle of this invention is explained. When blood is separated for every component by the centrifuge method, each component presents an original color, respectively. By recognizing a difference of this color from the red who incorporated from the color picture, green, and the shade information on blue (RGB), the boundary of a blood serum part and other parts is recognized among constituents of blood, and the amount of blood serums is calculated after this.
[0018] this invention persons analyzed the color picture of the blood collecting test tube after centrifugal separation. Consequently, the chromaticity information (mixing ratio of RGB each component to the average of RGB) which removed lightness information from the shade information on RGB could be searched for, and it has checked that the saturation value calculated from this chromaticity information showed a value high in a blood serum part.
[0019] While this looks a blood serum part is highly transparent and vivid as an image, a clot is the solid of a blackish color and image top vividness depends it on a low thing. Moreover, it is because a separating medium also presents opalescence and vividness looks low clearly compared with a blood serum part. Not only when it is the yellow which blood with the normal

color of a blood serum shows, but in the blood serum near red etc., it will be applied, and these things can calculate the field of a blood serum part in almost all blood collecting tubing by recognizing the field where saturation is high.

[0020] Drawing showing the concrete configuration of 1 operation gestalt of the amount measuring device of blood serums of this invention based on the above principles in drawing 1 and drawing 2 are the flow charts explaining the content of control of this equipment. In drawing 1 first, the amount measuring device 1 of blood serums of a blood collecting test tube Color CCD camera (only henceforth a camera) 2, and the video input board 3, A personal computer 4 and the motor 5 for a test tube revolution (only henceforth a motor), the glossiness sensor (for bar code label location detection) 6, the test tube chuck 7, and the lighting 8 for image photography -- since -- it is constituted, and gets down and a camera 2, a motor 5, the glossiness sensor 6, the test tube chuck 7, and lighting 8 are arranged in the measurement black box 9, respectively.

[0021] Here, said camera 2 carries out color photography of the blood collecting test tube 10, and constitutes a means to acquire the color image pick-up information on this blood collecting test tube 10. Moreover, red [in / in said personal computer 4 / each pixel of the color image pick-up from said color image pick-up information], Blue, a means to search for shade information [being green (RGB)], a means to remove lightness information from said RGB shade information, and to search for chromaticity information, A means to search for saturation information from said chromaticity information, a means to ask for the borderline location of a blood serum part and other parts among constituents of blood from said saturation information, The variation in each dot of the pixel of RGB each component is calculated from the pixel order predetermined dot near [for which it asked with a means to calculate the amount of a blood serum extractable from said boundary information, and said saturation] the borderline location. Variation has equipped the function by software as each means of the means which makes the location used as max a borderline location.

[0022] Moreover, a means set the blood-collecting test tube 10 constitutes, and a personal computer 4 has equipped by software the function as a control means which controls said motor 5 as a revolution means based on the detecting signal from the glossiness sensor 6 so that the location where the bar code label in which said motor 5, the glossiness sensor 6, and a personal computer 4 rotate the blood-collecting test tube 10, and the specimen ID of this blood-collecting test tube 10 front face is shown is not stuck may turn to bearing of the exposure axis.

[0023] Amount measurement processing of the blood serum part using this amount measuring device 1 of blood serums is performed as follows. That is, the blood collecting test tube 10 is placed in front of a camera 2, and it is made for the location where the bar code label which is made to rotate the blood collecting test tube 10, and shows Specimen ID to the transverse plane of a camera 2 by the motor 5 is not stuck to come.

[0024] In this case, although the location where the bar code label is not stuck on reverse by a bar code label's sticking and detecting a location is got to know, this is an approach generally used and said glossiness sensor 6 can perform it easily. When the blood collecting test tube 10 of the location where the bar code label is not stuck in front of the camera 2 is reflected, the color image of the blood collecting test tube 10 is incorporated on a video input board.

[0025] The image of the video input board 3 is captured in a personal computer 4 in a bit map format, 8 dot each each [of right and left] pixel is extracted from the center line of the perpendicular direction of a blood collecting test tube image to recognition, and it changes into the shade information on RGB in each pixel. Although 8 dots of right and left were used for the number of pixels to extract with this operation gestalt, it is what was adopted as an example of recognition count, and this invention itself is not limited to 8 dots of right and left. From the shade information on this RGB, recognition count of the boundary of the oil-level location of a blood serum part and a clot or a separating medium, and a blood serum part is carried out.

[0026] Next, with reference to the flow chart of drawing 2 , the detail of amount measurement processing of the above-mentioned blood serum part is explained based on the content of control of a personal computer 4.

[0027] It is step 1 (it is written as S1 by a diagram.) first. the following -- being the same -- it sets and a blood collecting test tube is positioned using the glossiness sensor 6. That is, a control side is ordered the location which does not have a bar code label using the glossiness sensor 6 so that it can incorporate without the image of the recognition field shown in drawing 3 overlapping the bar code label 11.
[0028] In step 2, the image pick-up information on the blood collecting test tube 10 is acquired with a camera 2. That is, if a personal computer 4 receives the control signal of the glossiness sensor 6, it will capture blood collecting test tube image pick-up information with a camera 2.
[0029] In step 3, blood collecting test tube information is transmitted to the video input board 3 as an NTSC video input signal from a camera 2.
[0030] At step 4, the information from the video input board 3 is changed into a well-known bit map format.
[0031] At step 5, it changes into the RGB shade information in each pixel from bit map format information.
[0032] At step 6, it is made the chromaticity information (r, g, b) which removed lightness information from RGB shade information.
Namely, r=R/(R+G+B)
g=G/(R+G+B)
b=G/(R+G+B)
r+g+b=1 It considers as a chromaticity flat surface.
[0033] Saturation information is searched for at step 7. That is, as shown in drawing 4 (A), chromaticity information (r, g, b) serves as a set of the point on the same flat surface (r+g+b=1 chromaticity flat surface), and asks for saturation from the point on a flat surface as shown in this drawing. Saturation is expressed with the ratio of WP/WQ in drawing 4 (B) (reference image-processing applied-technology Kogyo Chosakai Publishing). In this case, the point W of drawing 4 (B) expresses an achromatic color at the center of gravity of an equilateral triangle. Points P are a point on extension of Segment WP, and an intersection of point r+g+b=1 (r>=0, g>=0, b>=0) on a flat surface.
[0034] A borderline is recognized at step 8. That is, since a blood serum part has the value of saturation higher than other parts, it recognizes the part to be a blood serum. A detailed description of this asks for the moving average from the incorporation starting position of saturation information to termination. If this is expressed with a graph, it will become like drawing 5 . A blood serum part determines the default value which can surely be extracted, and recognizes an upper part as a blood serum part from the level determined with the default value. The maximum of |Rn+1-Rn |+|Gn+1-Gn |+|Bn+1-Bn | (refer to the graph of drawing 6) of the part is detected by making 15 dots into a recognition region around the pixel of the ends recognized as a blood serum part as an interface location of a blood serum front face or a blood serum, and a separating medium.
[0035] The capacity of a blood serum extractable for every classification of a blood collecting test tube is calculable by giving the classification of the photoed blood collecting test tube to the blood serum boundary information searched for according to the above operation.
[0036] In addition, when the rear-spring-supporter bar code label 11 is stuck more than the semicircle of the blood collecting test tube 10 by putting the lighting 8 to be used in the recognition of the amount of blood serums based on this saturation information from the transverse plane of the blood collecting test tube 10 and it sees from the transverse plane of a camera 2, if there is about 6mm or more of clearances where the bar code label 11 is not stuck, recognition of the amount of blood serums is possible.
[0037] Moreover, it can recognize with this configuration which recognizes a blood serum field based on saturation information, without being influenced of the alphabetic character which is photography by the reflected light and was printed by the bar code label, and a notation.
[0038] Furthermore, it is desirable to perform range correction processing for deducing the magnitude of a measurement object from a video image actually in addition to each above-mentioned processing. This range correction photos the points (for example, LED etc.) that distance became settled with the test tube image, always amends distance, and guarantees the

accuracy of measurement.

[0039] With the blood collecting test tube 10 which extracted direct blood from the patient by using the equipment which recognizes the boundary location of the blood serum capacity in the blood collecting test tube 10 and a clot or a separating medium, and a blood serum from a color picture with a camera 2 according to this configuration With the condition that the bar code label was stuck, since the amount of blood serums can be obtained before distributive pouring to accuracy, among the extracted blood, according to the extractable amount of blood serums, it becomes possible to assign a blood serum from analysis and inspection with high priority more, and a deployment of a precious blood serum is attained.

[0040] Moreover, it is not necessary to insert a sensor into the blood collecting test tube 10, and, therefore, sensor washing etc. can manufacture an unnecessary safe distributive-pouring machine easily by telling a distributive-pouring machine about the boundary location of a blood serum oil-level location and a clot or a separating medium, and a blood serum.

[0041] Furthermore, in this measuring device, the magnitude of a facility is small, there are few costs concerning plant-and-equipment investment, and they end, and the interface location detection time concerning one blood collecting test tube also has the advantage of being short.

[0042] In addition, in the above-mentioned configuration, the classification of the blood collecting test tube to be used can also be recognized from the incorporated color picture. Moreover, if the paper of the member of the white system which carried out abbreviation adhesion, for example, white, and light gray, or the body of plastics is placed behind the blood collecting test tube 10 seen from the camera 2 at the blood collecting test tube 10 The lightness of the blood serum image in the part on which the bar code label was stuck by the blood collecting test tube image, and the part on which a bar code label is not stuck, saturation, and the image that does not almost have the difference of a hue can be obtained, and it can consider as photography of blood collecting tubing which lessens effect of a bar code label.

[Translation done.]

DESCRIPTION OF DRAWINGS

[Brief Description of the Drawings]
[Drawing 1] Drawing showing the concrete configuration of 1 operation gestalt of the amount measuring device of blood serums of this invention
[Drawing 2] The flow chart explaining the content of control of equipment same as the above
[Drawing 3] Drawing showing the relation between the image of a recognition field, and a bar code label
[Drawing 4] For (A), (B) is drawing showing chromaticity information, and drawing showing saturation.
[Drawing 5] The graph showing the moving average from the incorporation starting position of saturation information to termination
[Drawing 6] | The graph showing Rn+1-Rn |+|Gn+1-Gn |+|Bn+1-Bn |
[Description of Notations]
1 The Amount Measuring Device of Blood Serums
2 Color CCD Camera
3 Video Input Board
4 Personal Computer
5 Motor for Test Tube Revolution
6 Glossiness Sensor
10 Blood Collecting Test Tube
11 Bar Code Label

[Translation done.]

* NOTICES *

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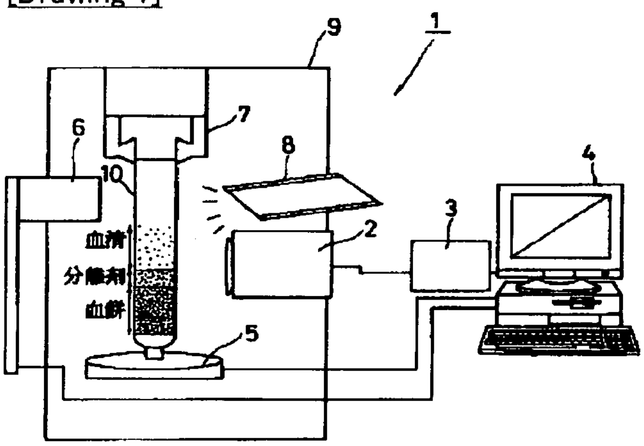
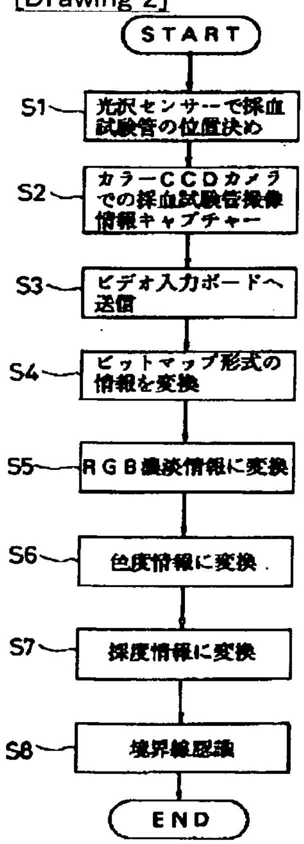
1.This document has been translated by computer. So the translation may not reflect the original precisely.
2.**** shows the word which can not be translated.
3.In the drawings, any words are not translated.

DRAWINGS

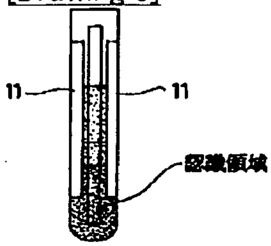
[Drawing 1]



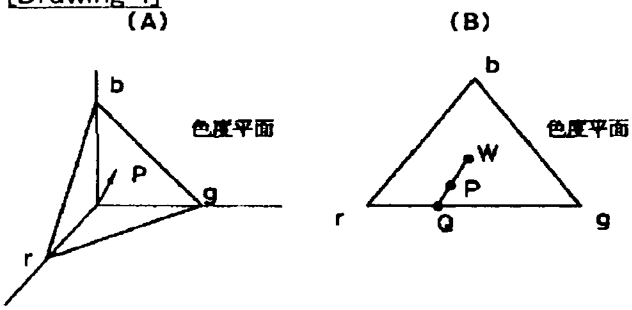
[Drawing 2]



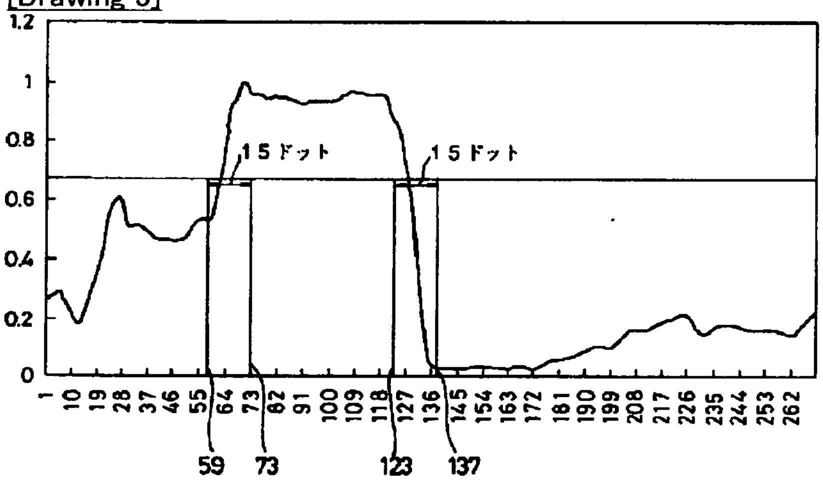
[Drawing 3]



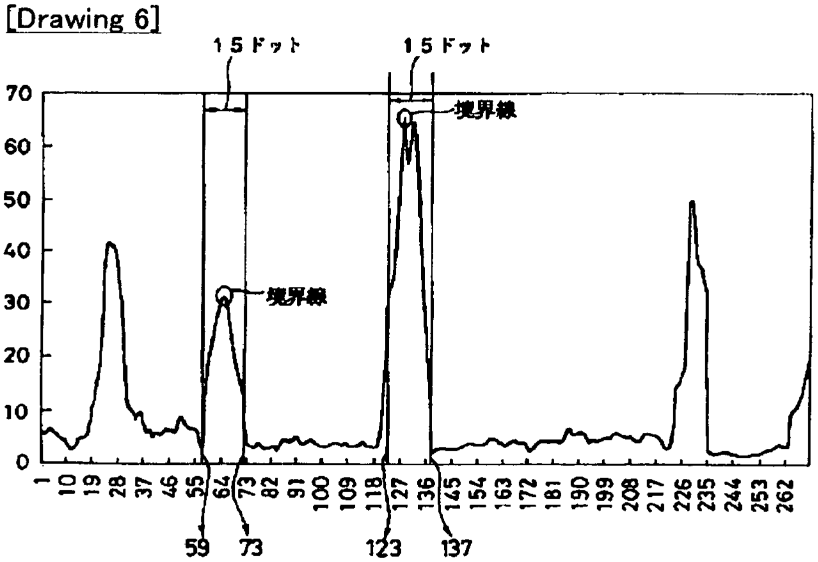
[Drawing 4]



[Drawing 5]



[Drawing 6]



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(71) Applicant: **HITACHI, LTD.**
6, Kanda Surugadai 4-chome
Chiyoda-ku, Tokyo 101(JP)

(72) Inventor: **Imai, Kazumichi**
Tsukubadai Apato 1-103, 663, Ichige
Katsuta-shi(JP)
Inventor: **Nomura, Yasushi**
991-15, Motoyoshidacho
Mito-shi(JP)
Inventor: **Koga, Masataka**
43-2, Taiseicho
Katsuta-shi(JP)
Inventor: **Tokinaga, Daizo**
42-10, Mejirodai-1-chome
Hachioji-shi(JP)
Inventor: **Takahashi, Satoshi**
Keimeiryo 209, 14-13, Honda-3-chome
Kokubunji-shi(JP)
Inventor: **Oki, Hiroshi**
8-1, Migimomi
Tsuchiura-shi(JP)
Inventor: **Miyake, Ryo**
Park de Kotten 139
Enschede(NL)
Inventor: **Okano, Kazunori**
17-2-402, Honcho-5-chome
Shiki-shi(JP)
Inventor: **Yasuda, Kenji**
52-5, Minamidai-4-chome
Nakano-ku, Tokyo(JP)

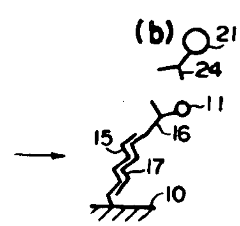
(74) Representative: **Strehl, Schübel-Hopf, Groening**
Maximilianstrasse 54 Postfach 22 14 55
W-8000 München 22(DE)

(54) Method for immunoassay and apparatus therefor.

(57) A method for immunoassay of a trace vital component is provided. Using fine particles as label or marker 21, the fine particles are captured on reaction solid phase 10 in proportion to an amount of analyte by a specific reaction such as antigen-antibody reaction. Then, the fine particles are liberated and the number of fine particles is counted to determine the amount of analyte. The solution to be assayed containing the liberated matters is introduced into flow cell 1 and pulse-like fluorescence emitted when the fine particles pass through a flux of laser light irradiated from the direction crossing the flow at the right angle is detected and the pulse is counted to count the number of fine particles 21.

The marker or label, i.e., the fine particles once captured on the reaction solid phase are liberated and then counted. Therefore, influence of the label non-specifically bound to the solid phase can be eliminated. By using the fine particles as the label and counting the number of the particles, detection having a high linearity can be realized even at a low concentration.

FIG. 4



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BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a method for immunoassay and an apparatus for immunoassay. More particularly, the present invention relates to a method for immunoassay which comprises labeling an analyte such as an antigen, an antibody or a hapten contained in a biological sample, introducing the labeled analyte to a flow cell and performing optical determination, and to an apparatus for the immunoassay.

Related Art Statement

For determining a trace substance in a biological sample such as blood, a method for immunoassay utilizing an antigen-antibody reaction is known. Japanese Patent Application KOKAI No. 1-187459 which is one of the prior art of this type discloses a method for immunoassay using an antigen-antibody reaction in combination with photoacoustic spectrometry.

That is, according to Japanese Patent Application KOKAI No. 1-187459, a sample containing an antigen as an analyte is charged in a reactor, onto the inside wall of which an antibody has been immobilized. Then, a suspension containing the liberated fine particles in the reactor is formed, via the steps of washing and removing the unreacted sample, adding a solution containing an antibody to which fine particles such as latex or an inorganic oxide has been bound, washing and removing an excess of the antibody and adding a liberating solution. The suspension is then transferred to a photoacoustic cell and exposed intermittently to a laser light to detect a change in pressure in the photoacoustic cell. A photoacoustic signal is thus obtained.

On the other hand, Japanese Patent Application KOKAI No. 63-214668 proposes a highly sensitive method for immunoassay. In the prior art, a labeled antibody obtained by binding microcapsules encapsulated with a fluorescent substance to an antibody is used. Any solid phase is not used in this prior art. The microcapsule-labeled antibody is added to a sample containing a cell to be assayed to effect immune binding. After washing by centrifugation, the suspension is charged in a sheath flow cell of flow cyto-meter. When the immune complex of the cell and the labeled antibody flows across a flux of laser light irradiated in the flow cell, fluorescence from the microcapsule is detected.

An example utilizing a sheath flow cell, though it is irrelevant to immunoassay, is disclosed in, e.g., Japanese Patent Application KOKAI No. 2-80937. According to the prior art, a sample container charged with stained blood cells is transferred to a sampling position and a blood cell suspension in the sample container is supplied to an inlet room of the sheath flow cell through a pipetting nozzle. The flow cell is so constructed that the flow cell is exposed to a laser light; when the stained cells move across a flux of the laser light, fluorescence and scattered light are detected.

In Japanese Patent Application KOKAI No. 1-187459 described above, a photoacoustic signal is detected and the signal obtained is dependent on the concentration of the whole suspension. Therefore, where an analyte has a very small concentration, it is difficult to determine the analyte with high precision. Where a labeled antibody is charged in a reactor with solid phase, the labeled antibody not only reacts specifically with the antigen bound to the solid phase but is also adsorbed non-specifically with the surface of the solid phase. Thus, this method involves a problem that the labeled antibody adsorbed non-specifically cannot be removed by ordinary washing operations. Where sodium hydroxide solution is used as a solution for liberating the marker as in Japanese Patent Application KOKAI No. 1-187459, however, both the specifically bound labeled particles and the non-specifically adsorbed labeled particle are liberated from the solid phase. Accordingly, photoacoustic spectrometry of a sample solution containing them results in causing serious measurement error, as a matter of course.

According to the method shown in Japanese Patent Application KOKAI No. 63-214668, it is unnecessary to use solid phase; the amount of signal detected from each of the labels becomes large, but washing is required using a centrifuging machine. Therefore, the operation is troublesome and automated only with difficulty. Further in Japanese Patent Application KOKAI No. 2-80937, no consideration is made to apply the method to immunoassay.

SUMMARY OF THE INVENTION

An object of the present invention is to provide a method for immunoassay which provides accurate results of measurements with high sensitivity even though an analyte is contained in a sample in a trace amount, and an apparatus for the immunoassay.

Another object of the present invention is to provide a method for immunoassay which can minimize as less as possible contamination of a particle label based on non-specific adsorption which did not bind to an analyte, and an apparatus for the immunoassay.

A further object of the present invention is to provide a method for immunoassay which can de-

termine a plurality of analytes using a plurality of reactors having a reaction solid phase of the same mode, and an apparatus for the immunoassay.

The present invention relates to a method for immunoassay which comprises the steps of:

(a) supplying a sample to a reactor having a solid phase, to which a receptor is immobilized, to bind an analyte in the sample to the receptor;
(b) removing the unreacted sample from the reactor and then supplying a receptor labeled with fluorescent particles to the reactor to bind the labeled receptor to the solid phase via the analyte;
(c) removing an excess of the labeled receptor from the reactor and then supplying a liberating reagent containing a label-liberating agent to the reactor;
(d) introducing the fluorescent particle-containing solution to be assayed, which has been liberated from the solid phase, into a flow cell;
(e) detecting fluorescence based on the fluorescent particles which pass through the flow cell to count the number of the particles detected; and,
(f) computing the concentration of analyte in the sample based on the number of particles counted.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows an outlined construction of one embodiment of the present invention.
Fig. 2 is a diagram for signal processing in two routes using the apparatus of Fig. 1.
Fig. 3 illustratively shows an embodiment of fixing or immobilizing a receptor onto a solid phase.
Fig. 4 illustratively explains the reaction state in a reactor.
Fig. 5 shows an example of calibration curve obtained using the apparatus of Fig. 1.
Fig. 6 shows an outlined construction of another embodiment of the present invention.
Fig. 7 is a drawing for explaining the procedure when CEA is measured with the apparatus of Fig. 6.
Fig. 8 shows comparison of the results when standard CEA sample was measured.

In the figures, numerals denote:

10
solid phase
11, 27
analyte (CEA)
15, 17
oligonucleotide
16, 26
receptor for solid phase (anti-CEA antibody)
21
fluorescent latex particle
23, 31
fluorescent particle
24, 24a, 29, 63
labeled antibody
25
restriction enzyme
32
turn table
33
sample container
35
reagent table
36a, 36b, 36c
container for labeled antibody solution
38
container for liberating reagent solution
42
movable arm
43
pipetting nozzle
46
controller
47
sheath flow cell
48
inlet for charging room
56
counter
61, 62
microplate
90a, 90b, 90c
container for antibody solution for fixing
94
tank for washing nozzle

DETAILED DESCRIPTION OF THE INVENTION

According to the present invention, the amount of a label associated with an antigen antibody reaction is counted as the number of the label, not in terms of its concentration. In order to avoid to count the labeled antibody non-specifically adsorbed to the solid phase, only the label bound by the antigen-antibody reaction is selectively liberated without liberating the non-specifically adsorbed labeled antibody, and only the liberated label is counted.

As the label in the present invention, fluorescent fine particles are preferably used. The method for immunoassay in accordance with the present invention comprises the steps of

supplying a sample to a reactor having a solid phase, to which a receptor is bound, to bind an analyte in the sample to the receptor;

removing the unreacted sample from the reactor, supplying a receptor labeled with fluorescent particles to the reactor to bind the labeled receptor to the solid phase via the analyte;

removing an excess of the labeled receptor from the reactor and then supplying a liberating reagent containing a label-liberating agent to the reactor;

introducing the fluorescent particle-containing solution to be assayed, which has been liberated from the solid phase, into a flow cell;

detecting fluorescence based on the fluorescent particles which pass through the flow cell to count the number of the particles detected; and

computing the concentration of analyte in the sample based on the number of particles counted.

A preferred embodiment of the present invention also comprises the following procedures, i.e., supplying a sample to a reactor having a solid phase, to which a receptor is bound via nucleic acid, polynucleotide or DNA, to immunologically bind an analyte in the sample to the receptor; supplying a labeled receptor to the reactor to bind the labeled receptor to the solid phase via the analyte; removing an excess of the labeled receptor from the reactor and then supplying a solution containing a restriction enzyme to the reactor to digest nucleic acid bound to the solid phase; introducing the label-containing solution to be assayed, which has been liberated from the solid phase, into a flow cell; and detecting the number of the label which passes through the flow cell.

As the receptor bound to the solid phase, where the analyte is an antigen or hapten, an antibody capable of specifically binding thereto is used; where the analyte is an antibody, an antigen capable of specifically binding to the antibody is used.

After a biological sample is supplied to the receptor with the solid phase to bind the analyte to the solid phase, a labeled antibody capable of immunologically binding to the analyte is added to the receptor. Thereafter, a solution containing an excess of the labeled antibody is removed from the receptor by suction, etc. By such operations for washing and removal, the labeled antibody non-specifically adsorbed to the surface of the solid phase is not split off but remains in the receptor.

Using the liberating reagent solution containing a specific label-liberating agent, only the antigen-antibody binding (immune complex) can be selectively dissociated, without substantially liberating (dissociating) the labeled antibody non-specifically adsorbed to the surface of the solid phase. Thus, only the label immunologically bound is selectively liberated into the solution in the receptor by supplying the liberating reagent solution. One of the specific label-liberating agent is a caotropic ion. As the caotropic ion, CCl_3COO^-, SCN^-, CF_3COO^- and the like may be used.

Herein, nucleic acid for binding an antibody or antigen as the receptor to the solid phase includes oligonucleotide. The antibody or antigen on the solid phase in the receptor has been bound to the solid phase via double stranded nucleic acid, when a sample is supplied.

In the case of assaying for multiple items, many receptors having the same mode are prepared and as the analytical operations are initiated, specific receptors allotted to the respective analytes are bound to the solid phase. That is, single stranded nucleic acid having the same mode in the base sequence is previously fixed to the solid phase in each receptor and single stranded complementary nucleic acid having the receptors corresponding to various analytes is added to the reactors, as allotted, which are designed to correspond to the respective analytes, to form double strand (hybrid) on the solid phase in each reactor, where various receptors are bound. By doing to, a series of receptors for assaying multiple items which may immunologically react with various analytes are prepared.

After the analyte in the sample is immunologically bound to the solid phase in the reactor bound to the solid phase through nucleic acid, a labeled receptor is further bound thereto. In this case, a restriction enzyme is used as a label-liberating agent for splitting the label off from the solid phase. As the restriction enzyme, there may be advantageously used hydrolase such as restriction endonuclease, phosphodiesterase, pyrophosphatase, peptidase, esterase, etc. Many other known restriction enzymes may also be used. By such restriction enzymes, the double-stranded DNA is cleaved at a definite site.

The solid phase in the reactor comprises inner wall surface of the reactor itself, e.g., a test tube or a microplate, or spherical materials such as glass balls encased in the reactor, the surface of which has been so treated that can chemically bind to the receptor to be bound.

As the label or marker, fluorescent fine particles are advantageously used. The fluorescent particles are practically latex particles or inorganic particles, on the surface of which a fluorescent substance layer is formed. Alternatively, a mixture of particle-forming composition and fluorescent substance is particulated and the resulting particles may be practically used. Fluorescence is detected by a flow cyto-meter and the number of particles passed through the flow cell is counted. In this case, a diameter of the fluorescent particle is 10^{-5} to 10^{-3} mm. Examples of the fluorescent substance which can be used for the fluorescent particles are fluorescein, coumarine derivatives, rhodamine derivatives, dansyl, umbelliferone, etc. which are all known.

Where nucleic acid is cleaved and the label is liberated, the label-containing substance led to the

flow cell is in such a state that a part of the cleaved nucleic acid, the receptor on the solid phase side, the analyte and the labeled receptor are combined with each other. On the other hand, nucleic acid bound to the receptor on the solid phase to which the analyte has not been bound is also cleaved with a restriction enzyme and led to the flow cell; however, since the label is not bound to such liberated matter, no fluorescence is detected in the flow cell so that measurement error does not occur substantially.

In the case of determining a concentration as in the prior art, the amount bound is counted as the statistical mean value. Therefore, detection sensitivity is insufficient at a low concentration and measurement data vary every sample by the influence of interferants contained in the sample so that accurate measurement data are not obtained. In addition, in order to avoid the influence of non-specific adsorption, it is attempted to perform the assay after dissociating the antibody-antibody complex; in this case, an additional problem encounters that a composition of the dissociation solution does not fit the conditions for enzyme reaction so that the dissociated sample cannot be measured as it is. By the procedures for dissociation and liberation, the solution to be assayed is diluted so that the lower limit for quantitative determination substantially goes up.

According to the method of counting the particles, even only one particle can be counted and hence, a detection sensitivity sufficient to measure an antigen of a low concentration can be obtained. That is, each particle gives an amplified signal. Using the particles as a label or marker, the number of the particles bound by antigen-antibody reaction is counted. Therefore, the count value directly reflects the amount of antigen.

According to the method of labeling fine particles and counting the particles one by one, it is designed to count a signal between two p.d. levels (high and low) on a predetermined definite level, even though interferants cover the surface of the particles to reduce or amplify the fluorescent intensity. By doing so, influence of the interferants can be avoided and the particles can be counted accurately. The counting is not affected by noise in the measurement system. Since the absolute number of the particles can be counted, there is no influence of dilution. The particle number can also be counted in the dissociation solution without modifying its composition. Therefore, no additional care is needed for measurement.

In the atmosphere, many dusts below several microns are present and these dusts contaminate the solution to be assayed, as foreign particles. In the method which comprises irradiating a sample with light and measuring the light scattered by the sample, the desired labeled particles are not distinguished from the foreign particles and the amount of foreign particles increases the background concentration. Turning to the method of the present invention for measuring fluorescence, foreign particles having a strong fluorescence are absent in normal environment and the measurement is thus made without any error. Therefore, detection can be made with high sensitivity by the method of the present invention for counting the particles one by one, using the fluorescent particles.

In nucleic acid, in the case of, e.g., DNA, constituent unit, deoxyribonucleotide is bound to 3',5'-phosphodiethyl ester. On the other hand, protein which constitutes the major part of an antibody is a polymer of amino acids by polypeptide bond. Since the binding mode of nucleic acid is quite dissimilar to that of protein, there is the least possibility that protein might be denatured or degraded under reaction conditions for digesting nucleic acid. In particular, when endonuclease called a restriction enzyme is used, the cleavage occurs only at a specific nucleotide sequence of nucleic acid. Therefore, only the nucleic acid portion can be selectively cleaved, without cleaving the protein portion in the conjugated matter.

Accordingly, an antibody is bound to the solid phase via nucleic acid, an antigen and a labeled antibody are captured by antigen-antibody reaction; after washing, selective release can be made at the nucleic acid portion. In this case, only the label participated in the desired antigen-antibody reaction can be split off from the solid phase, without denaturing or degrading the antibody or enzyme. The non-specifically adsorbed label not by the antigen-antibody reaction is directly adhered to the solid phase not via the nucleic acid. Such a label is not liberated by the action of restriction enzyme. Thus, the influence of non-specific adsorption is eliminated and the amount of antigen can be determined accurately. Quantitative nature is not damaged, either.

Among enzymes which digest DNA (deoxyribonucleic acid), a restriction enzyme functions to recognize only a specific nucleotide sequence and cleave the specific sequence. The nucleotide sequence is called recognition cleave sequence and inherent to each restriction enzyme. Accordingly, where the recognition cleavage sequence of restriction enzyme used is inserted at at least one site in the nucleotide sequence of polynucleotide or oligonucleotide used for the binding, the restriction enzyme can be acted thereon to effect cleavage. The nucleotide sequence satisfying the condition can be freely set forth.

A reactor having a solid phase, to which a receptor such as an antibody is bound, is extremely advantageous for an immunoassay as stat-

ed hereinbefore, and therefore may be widely used for various immunoassays. That is, the reactor may be also used for a conventional immunoassay wherein an enzyme is used as a label or wherein instead of flow cell, a label is detected by conventionally measuring an optical absorbance. Thus, according to the other aspects of the present invention, there is also provided a method for immunoassay which comprises the steps of; supplying a sample to a reactor having a solid phase, to which a receptor is bound via nucleic acid, to bing an analyte in the sample to the receptor; removing the unreacted sample from the reactor and then supplying a label to the reactor to bind the label to the solid phase via the analyte; removing an excess of the label from the reactor and then supplying a restriction enzyme to cleave the nucleic acid; and detecting the label which has been liberated from the solid phase.

In the above method, as the label, there may be used an enzyme such as a peroxidase, and a fluorescence substance such as fluorescein and coumarine derivatives. The liberated label may be detected by utilizing an enzyme reaction. A conventional procedure may be used in the above immunoassay, as it is.

As a kit for the above immunoassay, there is provided a kit comprising a reactor having a solid phase, to which a receptor is bound via nucleic acid; and a restriction enzyme.

The first embodiment of the present invention is described by referring to Figs. 1 to 5.

Fig. 1 shows an outline of the entire construction of the apparatus for immunoassay which is an embodiment of the present invention. A series of containers may be placed in two lines on turn table 32. A plurality of sample containers 33 encasing blood samples are circularly arranged at the inner circumference and a plurality of reactors 34 each having a solid phase are circularly arranged at the outer circumference. As later described, single stranded complementary oligonucleotide is fixed on the solid phase of each reactor 34. The turn table 32 is rotated by pulse motor 92 operatively controlled by controller 46 to stop the desired sample container 33 and reactor 34 at the position of charging through pipetting nozzle 43. The line of the reactors is constructed to move in a thermostat.

A variety of reagent containers necessary for analytical operations are placed on reagent table 35. That is, containers 90a, 90b and 90c in which solutions of antibodies for fixing corresponding to various items A, B and C for analysis are charged, containers 36a, 36b and 36c in which solutions of fluorescent particle-labeled antibodies corresponding to the various items for analysis are charged, container 38 in which a liberating reagent solution containing a restriction enzyme is charged, and other necessary buffer containers, etc. are held on reagent table 35. The pulse motor 93 operatively controlled by controller 46 rotates reagent table 35 by a desired angle and positions the instructed reagent container at the position of sucking through nozzle 43, with a necessary timing.

Automated pipetting mechanism 39 is equipped with pipetting nozzle 43 mounted to movable arm 42, rotary driving part 45 for horizontally rotating arm 42, vertical driving part 44 for vertically moving arm 42, syringe pump 40 connected with nozzle 43 via tube 41, and washing solution tank 37 which also acts as an extrusion solution. With the movement of arm 42, pipetting nozzle 43 can rotate on the two charging positions on turn table 32, the position of receiving reagent on reagent table 35, inlet 48 for room of flow cell 47 and nozzle washing tank 94, as a rotary radius. The nozzle can go down and go up at the respective positions.

The inside of sheath flow cell 47 is constructed as in that used for known flow sight meter, but a reagent charging room equivalent to that shown in Japanese Patent Application KOKAI No. 2-80937 is opened at the upper portion. Therefore, nozzle 43 can enter into charging room inlet 48 and discharge the solution to be assayed into sheath flow cell 47. By feeding pump 9, the sheath solution in sheath solution tank 6 is fed at a constant flow amount, flows along the inner wall of flow cell 47 and discharged in discharge reservoir 95. The solution to be assayed which is introduced into the flow cell flows at the center of the flow of the sheath solution.

A laser light source 49 can emit an argon laser having an oscillation wavelength of 488 nm. After its beam width is expanded by beam expander 50, a flux of the laser light is irradiated on sheath flow cell 47 to stop down by lens 51 and focus on the flow of the solution to be assayed. For condensing the fluorescence from flow cell 47, objective lens 52 for microscope is used. Space filter 54 and wavelength selection filter 55 are provided in front of photomultiplier 53 as a photoelectric detector to remove scattered light and Raman scattering light. After the output of photomultiplier 53 is amplified by preamplifier 18, the output is amplified by linear amplifier 19 and noise is removed by lower pulse height analyzer 20a and upper pulse height analyzer 20b. Thereafter, the pulse between the two threshold values is integrated.

A high voltage is applied to photomultiplier 53 via transducer 96 and high voltage electric source 97. Sample numbers, results of counting, calibration curve, histogram for measuring fluorescence, etc. are output on display 57, printer 22 and floppy disk 58. Also via interface 59, they may be communicated with a personal computer.

A diagram for signal processing is shown in Fig. 2. Via circuit 20 for analyzing the output 80 of preamplifier by pulse height analyzer, pulse train 82 is obtained. The pulse train is integrated with counter 56. In another circuit for signal processing, output 80 is passed through levelling circuit 84 to convert the pulse number into voltage change to obtain signal 85. The voltage change is counted with volt meter 86. The counting with this levelling may also be conducted by connecting with, e.g., a pen recorder and reading the recorded value.

Examples of the items determined by the apparatus shown in Fig. 1 are carcinoembryonic antigen (CEA, C-reactive protein (CRP) and α-fetoprotein (AFP). In this case, a solution containing anti-CEA antibody bound to single stranded oligonucleotide is charged in container 90a for antibody solution for fixing on reagent table 35; a solution containing anti-CRP antibody similarly bound is charged in container 90b for antibody solution for fixing; and, a solution containing anti-AFP antibody similarly bound is charged in container 90c for antibody solution for fixing. In containers 36a, 36b and 36c for labeled antibody solution, anti-CRP antibody, anti-digoxin antibody and anti-AFP antibody labeled with fluorescent fine particle, respectively, are charged.

The process of immuno-binding in each item for assay is similar; therefore, taking the case of CEA as an example, the process for analytical operations in the reactor is explained hereafter. The reactors 34 on turn table 32 all take the same mode prior to the procedures for assay. That is, as shown in Fig. 3, the inner wall of glass-made reactors 34 forms solid phase 10 and single stranded oligonucleotide 15 is fixed to the solid phase. The items for analysis are specified by the kind of antibody for fixing which is charged after the operation has started. Therefore, the same oligonucleotide 15 is fixed to each of the reactors 34 prior to initiation of the operations. Oligonucleotide 15 is bound to the solid phase having an amino group as the surface active group.

On the other hand, a solution containing antibody for fixing, in which anti-CEA antibody is bound to synthetic oligonucleotide having nucleotide sequence AGGCCT by maleimide thiol coupling and the sequence can be cleaved with restriction enzyme HaeI, is prepared, wherein, A, G, T and C are constituent units of nucleotide and denote adenine, guanine, thymine, and cytosine, respectively.

When the procedures for analysis are initiated, the antibody is dispensed from container 90a for antibody solution through nozzle 43. By discharging in the corresponding reactor 34, it is specified that the reactor is for assaying CEA. As shown in Fig. 3, oligonucleotide 17 in anti-CEA antibody 16 is bound to oligonucleotide 15 having the complementary strand in the solid phase side. Accordingly, this reactor 34 can recognize CEA.

Where the thus treated reactor is prepared prior to initiation of the operations for analysis, the reactor may be the one having a reactive solid phase capable of recognizing each item for analysis. Also for specifying other items, such can be effected simply by changing the solution of antibody for fixing.

In the thus prepared reactor 34, a sample dispensed from sample container 33 through nozzle 43 is charged. As shown in Fig. 4 (a), CEA 11 in the sample is bound to anti-CEA antibody 16 fixed onto solid phase 10. After reacting for 10 minutes, the unreacted sample from reactor 34 is sacked through nozzle 43 and discharged to washing tank 94. Then, buffer is added to reactor 34 followed by sucking and discharging through nozzle 43. This washing operation is repeated several times.

Subsequently, as shown in Fig. 4 (b), a solution containing anti-CEA antibody labeled with latex particles 21 which contain a coumarine derivative as a fluorescent substance is sucked from container 36a on reagent table 36a in a definite volume through nozzle 43 and discharged into the corresponding reactor 34. The reactor 34 is maintained at a definite temperature (37 °C) for a definite time period (15 minutes) on the turn table to perform the reaction, whereby labeled anti-CEA antibody 24 is bound to CEA 11 and fixed onto solid phase 10. In this case, as shown in Fig. 4 (c), a part of labeled anti-CEA antibody 24a which was not immunologically bound to CEA 11 is directly adsorbed onto the surface of solid phase 10. The solution containing an excess of the labeled antibody in reactor 34 is sucked and discharged into washing tank 94, through nozzle 43. Thereafter, the washing solution is discharged into the reactor through nozzle 43 and the operation for sucking and discharging the washing solution is further repeated.

Then, container 38 for liberating reagent solution is located at the sucking position by rotating reagent table 35. The solution containing restriction enzyme Hae I 25 in the container 38 is sucked in a definite volume through nozzle 43 and discharged into the corresponding reactor 34 on turn table 32. As shown in Fig. 4 (c) and (d), the double-stranded oligonucleotide bound to solid phase 10 is cleaved at the digesting site in the reactor and the liberated substance is suspended in the solution in reactor 34. Also for such liberation, a definite period of time (15 minutes) is required. By cleaving the specific hybrids of complementary nucleic acid or double-stranded DNA in the middle with restriction enzyme 25, the two liberated substances are present in the reactor. One of them is the

fluorometrically detectable liberated matter composed of antibody 24 with label 21, antigen 11, antibody 16 for fixing and restricted fragment of oligonucleotide, and another is the fluorometrically non-detectable isolated matter composed of the antibody for fixing which remains on the solid phase without causing immunological binding, and restricted fragment. The solution to be assayed which contain these liberated matters is sucked through pipetting nozzle 43 and discharged into sheath flow cell 47. Upon liberation with restriction enzyme 25, labeled antibody 24a directly adsorbed onto solid phase 10 is not liberated so that such does not contaminate the solution to be assayed.

By using as latex particles 21 containing a coumarine derivative the particles having a particle diameter of 0.1 μm and CEA standard sample instead of the sample described above, a calibration curve is obtained and shown in Fig. 5. The calibration curve has high linearity for quantitative determination and is excellent especially for quantitative determination at a low concentration. No curve observed with the assay using enzyme reaction at a low concentration was noted in this case. The lower limit of detection was determined by contrasting one particle to one molecule; it was below 10^{-22} mol as the absolute amount and below 10^{-18} mol/l as the concentration.

In the apparatus shown in Fig. 1, even in the case of assaying for a plurality of items, reactor 34 placed on turn table 32 may be the one equipped with the solid phase of the same mode. When the relationship between a sample and items to be assayed is input from an input device to controller (computer) 46, controller 46 controls the operation for each part of the apparatus to perform measurement of necessary items in a desired order. For example, where items are desired to be assayed in the order of A, A, B, C and B, the antibody for fixing is charged in each reactor in the order of 90a, 90a, 90b, 90c and 90b and fixed. The controller 46 also controls dispersed samples to perform measurement in an optional order. A sample is diluted, if necessary and desired.

After the sample is reacted, reactor 34 is washed and labeled antibodies 36a, 36b and 36c are then dispensed. The labeled antibodies are those obtained by binding labels to antibodies corresponding to the items A, B, and C to be assayed and therefore, the labeled antibodies are also automatically chosen by controller 46 in the order of 36a, 36a, 36b, 36c and 36b, according to the order of A, A, B, C and B initially designated.

Next, a second embodiment of the present invention is explained by referring to Figs. 6 to 8. In the apparatus for immunoassay shown in Fig. 6, the inner wall of microcapsule is used as the solid phase and X, Y moving mechanism is used in place of the turn table in the first embodiment.

In Fig. 6, microtiterplate 61 for reaction, microtiterplate 62 for sample, labeled antibody 63, liberating solution 64 and washing solution 65 are placed on the upper surface of the apparatus. The reagent part is kept cool with cooler 66. The reaction part is kept at 37°C with heater 67. The reaction part and labeled antibody 63 are designed to be shaken with vibrator 68.

As light source 69, He-Ne laser light having an oscillation wavelength of 632.8 nm is used. A light shielding plate 70 is provided so as to avoid incident light from the outside.

Laser light is shaped through prism 71 and condensed by lens 72. Fluorescence is condensed by lens 52, passed through spatial filter 54 and interference filter 55, and then detected with semiconductor optical detector 73. After amplifying with amplifier 74 and selecting the signal with pulse height analyzer 20, the signal is input into counter 56. Data processing is conducted with computer 46, which is connected with interface 59 to also communicate with outer equipments.

IN addition to a printer, output is also made into IC memory card 75. Initial setting of the apparatus is also memorized on the IC card to make it easy to start.

As the fluorescent fine particles, particles having a diameter of 0.1 and 0.2 μm containing methylene blue are used.

In order to avoid adverse affect of bubble contained in the sheath solution, etc., deaerators 76 and 77 are mounted.

For feeding the sheath solution and the solution to be assayed, a pneumatic pump may be constructed by pressurized air and such may be used for this purpose, instead of syringe pump 40. In this case, there is an effect of reducing pulse flow.

The labeled antibody obtained by binding an antibody to commercially available fluorescent fine particles is prepared and CEA is immunologically assayed, using the apparatus shown in Fig. 6. This embodiment is explained by referring to the illustrated drawing of Fig. 7.

Anti-CEA antibody is bound to fluorescent particles 23 (containing fluorescein; particle diameter of 0.1 μm, Molecular Probe Inc.), on the surface of which carboxy groups are introduced, using water-soluble carbodiimide.

Anti-CEA antibody 26 is bound to polystyrene-made microplate 61, which is used as the reactive solid phase.

Prior to the operations for measurement, CEA standard solution is prepared, which is made sample 27. Sample 27 is added to each well 28 of microplate 61 fixed with anti-CEA antibody 26, which is allowed to stand at room temperature for a definite period of time. After washing, labeled anti-

body 29 labeled with fluorescent particles 23 is added thereto to react them for a definite period of time. After thoroughly washing, a caotropic liberating solution 64 is added to liberate the fluorescent particles 31 captured onto the microtiterplate by antigen-antibody reaction. The solution containing the liberated fluorescent particles is made a solution to be assayed and the number of fine particles in the solution is counted. A calibration curve for CEA concentration is shown in Fig. 8, a.

For purpose of comparison, a calibration curve is prepared using as a label horse radish peroxidase (POD) which is one of enzyme, in the method for detecting the amount of POD by reacting with a fluorescent substrate. In this case, the label (POD) is liberated after the immune reaction, transferred to a separate microplate and subjected to enzyme reaction (shown by b. in Fig. 8). In addition, the label is not liberated but subjected to enzyme reaction in the same microplate as it is and then fluorometrically measured (shown by c. in Fig. 8).

It is obviously noted that the results a. according to the process of the present invention provide a lower background concentration and higher detection sensitivity at a low concentration.

In the fine particle counter explained in Embodiment 1, attention is paid only to the pulse component of the signal obtained by photoelectric amplifier, and this pulse number is counted. With a sample having a low density in the particle number, measurement data showing extremely high quantitative nature are obtained, as has already been explained. However, with a sample having a high density of the particle number, more than 2 particles pass across the flux of laser light which might result in failing to count and thus reduction in counting rate. On the other hand, with a sample of high concentration, the fluorescent intensity becomes strong so that the fluorescent intensity namely, the signal may be levelled with a definite time constant; even if its series component is counted, the measurement value in proportion to the concentration is given. Accordingly, if both the pulse number and the series component value are simultaneously measured, the region of concentration to be assayed can be broadened. By adopting such a process, the sensitivity of immunoassay can be increased by about two figures.

According to the present invention, accurate measurement results with high sensitivity can be obtained even though the analyte is contained in a sample in a trace amount. Therefore, the present invention greatly contributes to immunoassay.

Claims

1. A method for immunoassay which comprises the steps of:

   (a) supplying a sample to a reactor having a solid phase, to which a receptor is bound, to bind an analyte in the sample to the receptor;

   (b) removing the unreacted sample from the reactor and then supplying a receptor labeled with fluorescent particles to the reactor to bind the labeled receptor to the solid phase via the analyte;

   (c) removing an excess of the labeled receptor from the reactor and then supplying a liberating reagent containing a label-liberating agent to the reactor;

   (d) introducing the fluorescent particle-containing solution to be assayed, which has been liberated from the solid phase, into a flow cell;

   (e) detecting fluorescence based on the fluorescent particles which pass through the flow cell to count the number of the particles detected; and,

   (f) computing the concentration of analyte in the sample based on the number of particles counted.

2. A method for immunoassay according to claim 1, wherein said receptor is an antibody and said analyte is an antigen or hapten.

3. A method for immunoassay according to claim 1, wherein said fluorescent particles are latex particles or inorganic particles, on the surface of which a fluorescent substance layer is formed, or particulated a mixture of particle-forming composition and a fluorescent substance.

4. A method for immunoassay according to claim 1, wherein the particle diameter of said fluorescent particle is 10^{-5} to 10^{-3} mm.

5. A method for immunoassay according to claim 1, wherein said liberating agent contains caotropic ions.

6. An apparatus for immunoassay which performs operations for immunoassay according to claim 1.

7. A method for immunoassay which comprises the steps of:

   (a) supplying a sample to a reactor having a solid phase, to which a receptor is bound via nucleic acid, to immunologically bind an analyte in the sample to the receptor;

   (b) supplying a labeled receptor to the reactor to bind the labeled receptor to the solid

phase via the analyte;
(c) removing an excess of the labeled receptor from the reactor and then supplying a solution containing a restriction enzyme to the reactor to cleave nucleic acid bound to the solid phase;
(d) introducing the label-containing solution to be assayed, which has been liberated from the solid phase, into a flow cell; and
(e) detecting the number of the label which passes through the flow cell.

8. A method for immunoassay according to claim 7, wherein said nucleic acid has double-stranded DNA, or specific hybrids of complementary nucleic acid.

9. A method for immunoassay according to claim 7, wherein said nucleic acid is oligonucleotide.

10. A method for immunoassay according to claim 7, wherein said solid phase comprises a spherical substance encased in said reactor or the inner wall of said reactor, which is subjected to surface treatment that is chemically bound to said receptor.

11. An apparatus for immunoassay for which performs operations for immunoassay according to claim 7.

12. A method for immunoassay which comprises the steps of:
(a) preparing a plurality of reactors having a solid phase to which one of single-stranded nucleic acid capable of forming a hybrid is bound;
(b) supplying a first receptor to which another single-stranded nucleic acid capable of complementarily binding to said one single-stranded nucleic acid is bound to a first reactor among said plurality of reactors, wherein said first receptor is capable of specifically binding to a first analyte in a sample;
(c) supplying a second receptor to which the same as another single-stranded nucleic acid is bound and which is capable of specifically binding to a second analyte to a second reactor amount said plurality of reactors; and
(d) supplying a sample to said first and second reactors to proceed the immunoreaction of said first analyte in said first reactor and proceed the immunoreaction of said second analyte in said second reactor.

13. A method for immunoassay according to claim 12, characterized by supplying a labeled receptor to said reactors after the antigen-antibody reaction, removing an excess of the labeled receptor from said reactors and then supplying a solution containing a restriction enzyme to said reactors, and introducing the solution containing the label liberated from said solid phase into a flow cell as a solution to be assayed.

14. An apparatus for immunoassay for performing a method for immunoassay according to claim 13.

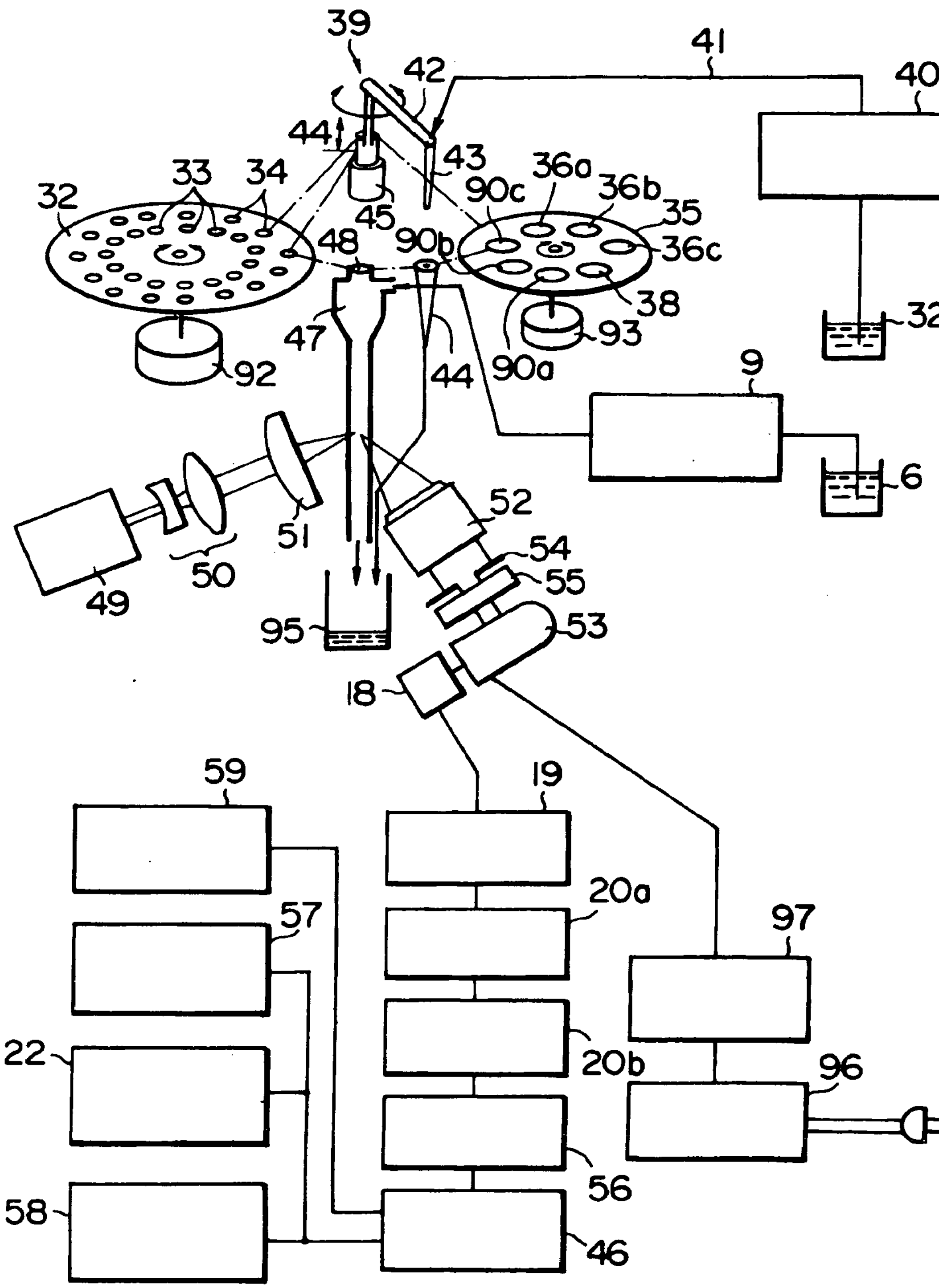
15. A method for immunoassay which comprises the steps of:
supplying a sample to a reactor having a solid phase, to which a receptor is bound via nucleic acid, to bind an analyte in the sample to the receptor;
removing the unreacted sample from the reactor and then supplying a label to the reactor to bind the label to the solid phase via the analyte;
removing an excess of the label from the reactor and then supplying a restriction enzyme to cleave the nucleic acid; and
detecting the label which has been liberated from the solid phase.

16. A method for immunoassay according to claim 15, which comprises the steps of:
binding a particle label to a solid phase in a reactor via nucleic acid;
supplying a restrction enzyme to said reactor to cleave said nucleic acid; and
introducing into a flow cell the particle label which binds a part of nucleic acid after said cleavage.

17. A reactor having a solid phase at the inner wall thereof, in which a receptor is bound to said solid phase via nucleic acid.

18. A kit for immunoassay which comprises:
a reactor according to claim 17; and
a restriction enzyme.

FIG. 1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33
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44
93
90a
92
9
52
6
51
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20b
96
56
58
46

FIG. 2

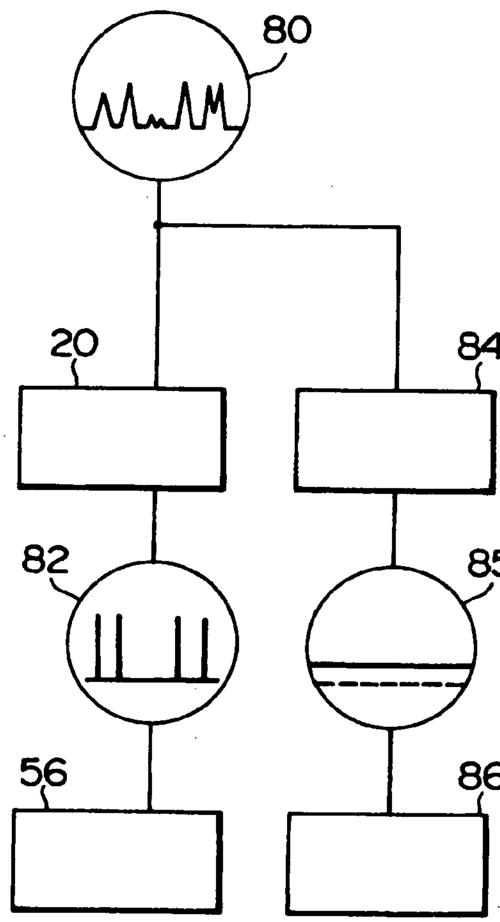


FIG. 3

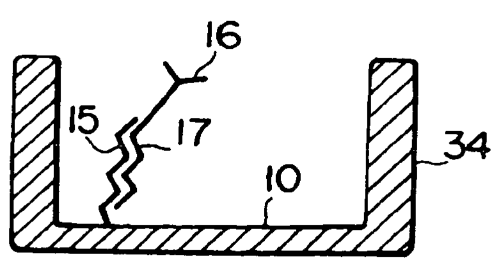


FIG. 4

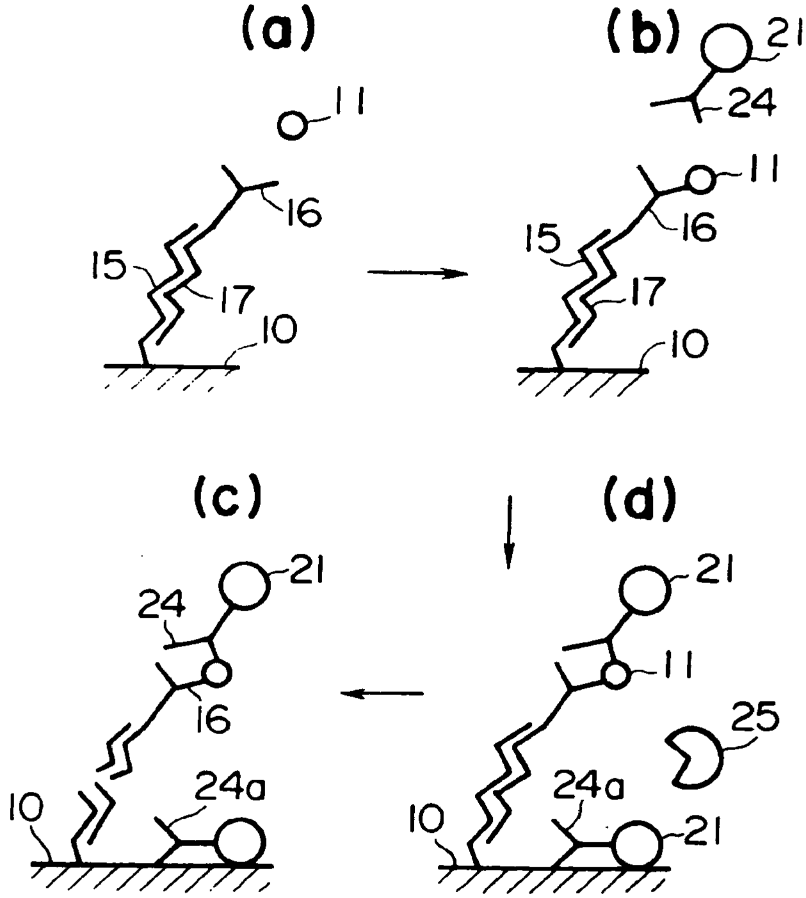
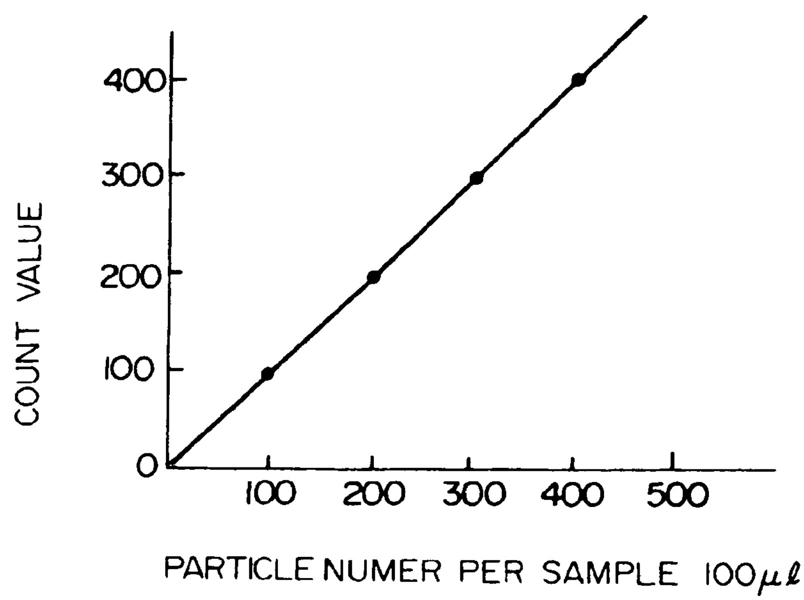

(a)
(b)
11
21
24
16
15
17
10
(c)
(d)
24a
25

FIG. 5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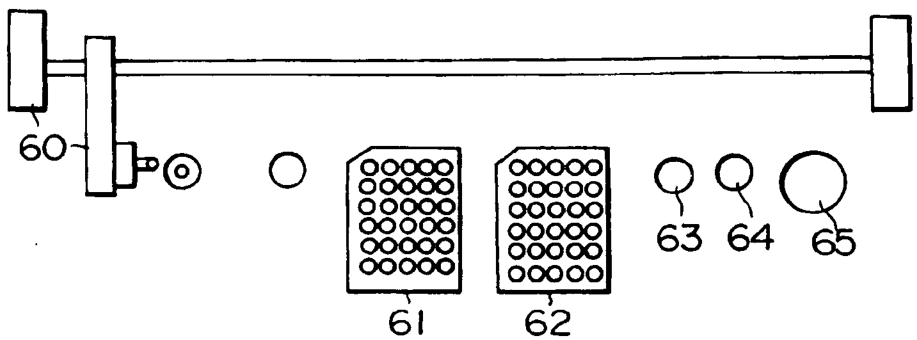
300
200
100
0
100
200
300
400
500
PARTICLE NUMER PER SAMPLE 100μl

FIG. 6A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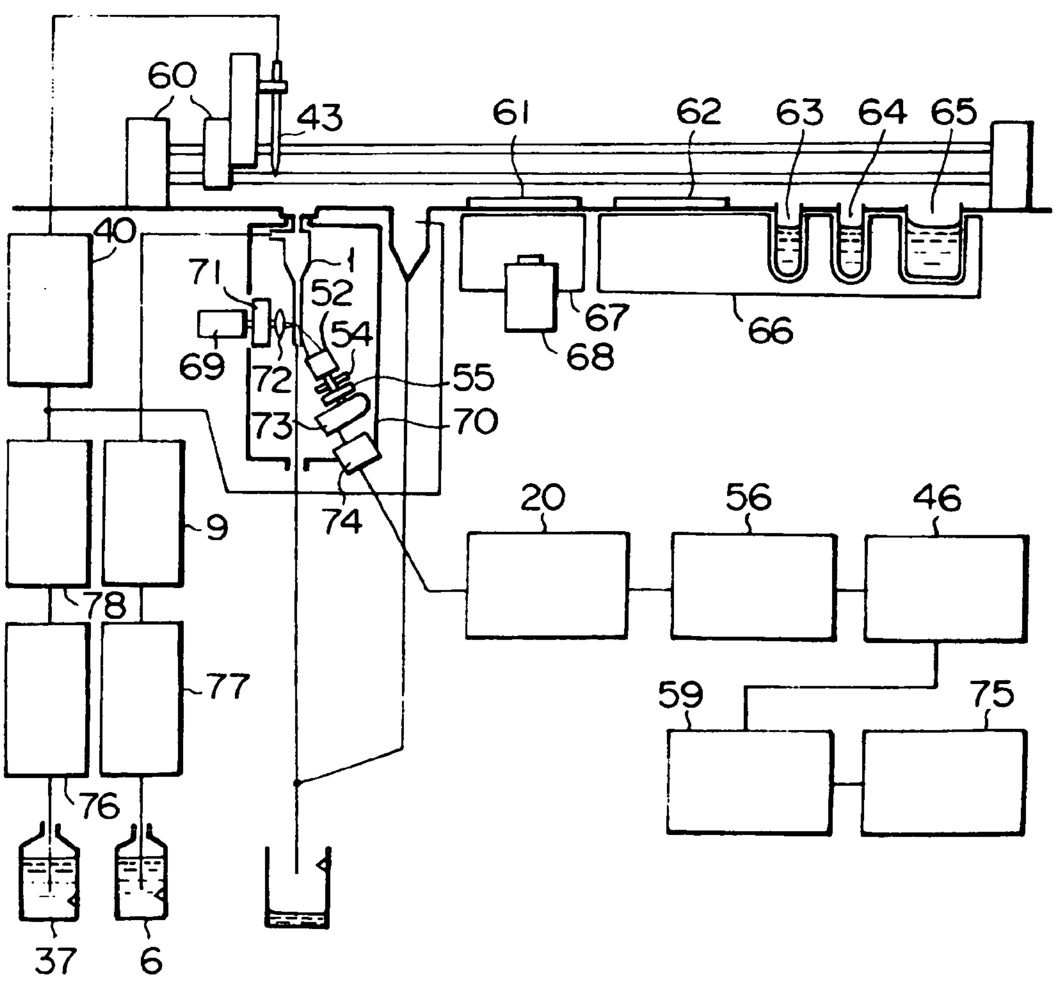
61
62
63
64
65

FIG. 6B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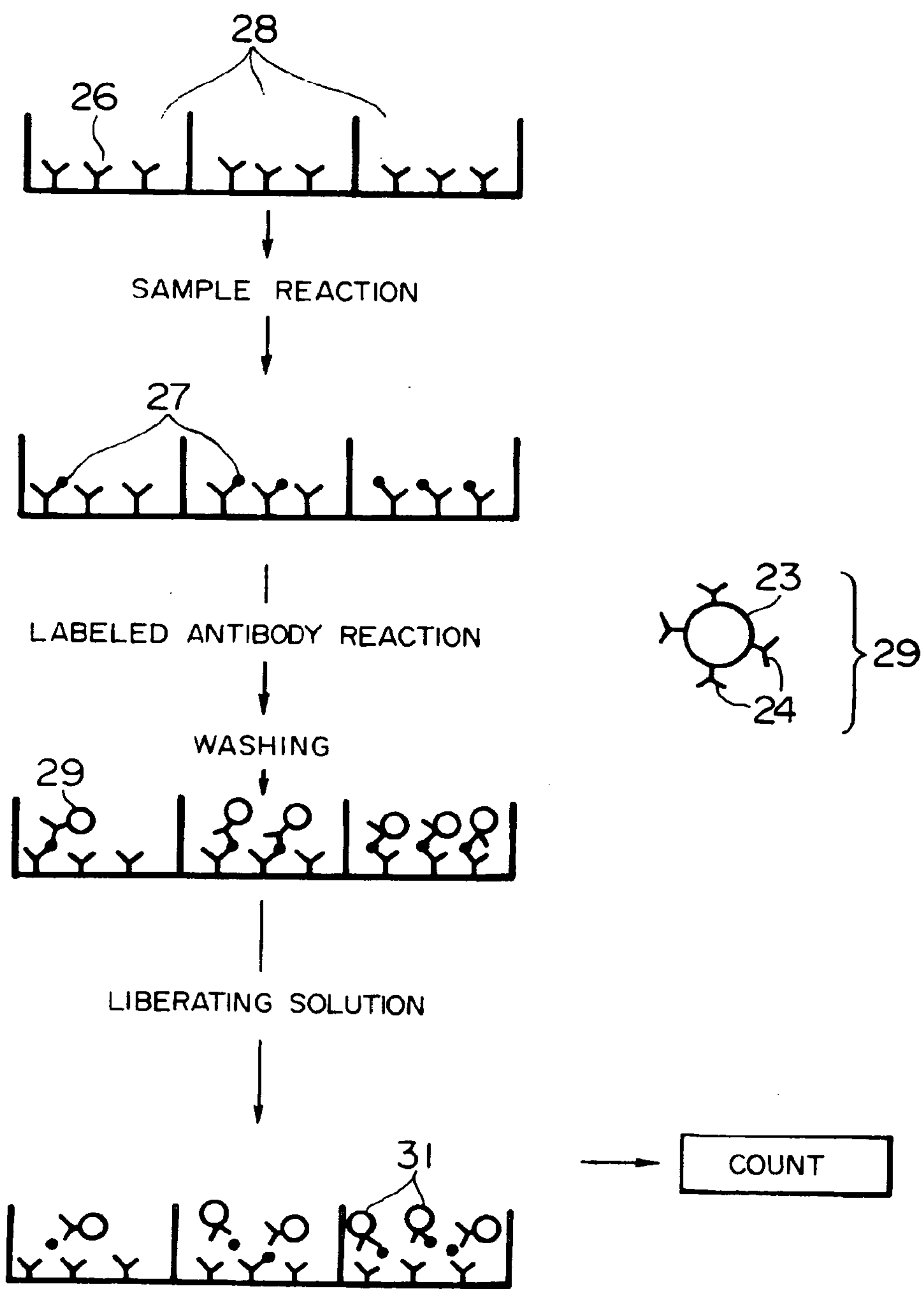
6
20
56
46
59
75

FIG. 7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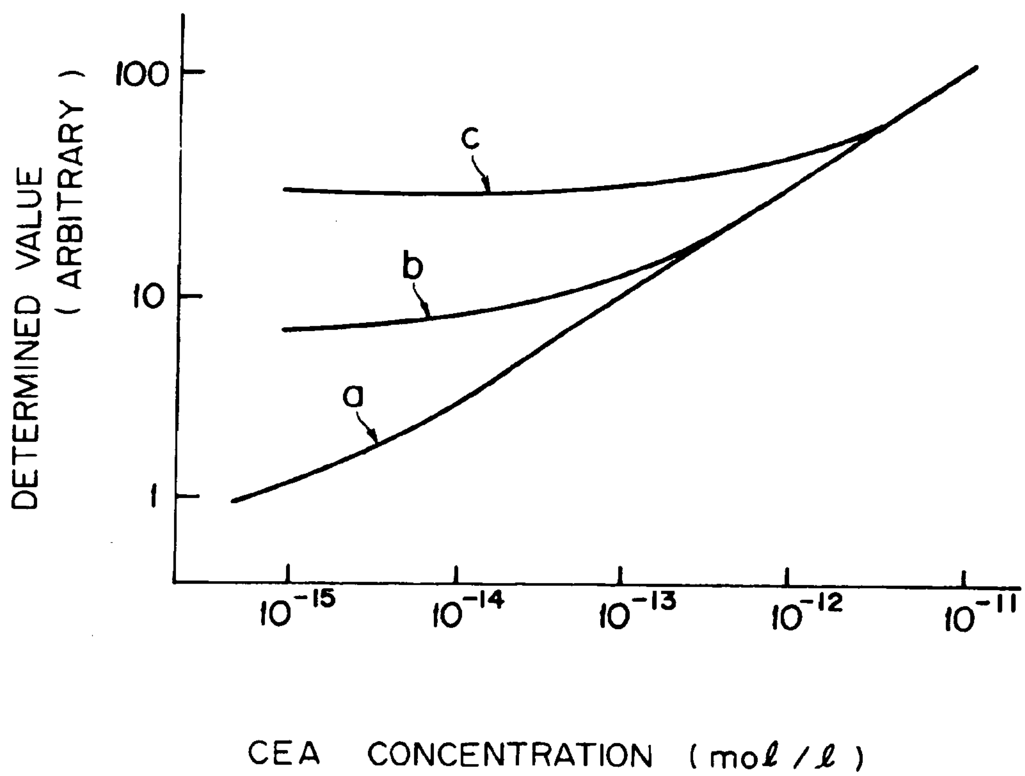
26
SAMPLE REACTION
27
23
LABELED ANTIBODY REACTION
29
24
WASHING
29
LIBERATING SOLUTION
31
COUNT

FIG. 8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(ARBITRARY)
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10^{-14}
10^{-13}
10^{-12}
10^{-11}
CEA CONCENTRATION (mol / l)